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Aromatic Amines: An Assessment of the Biological and Environmental Effects

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AROMATIC AMINES: An Assessment of the Biological and Environmental Effects

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Aromatic amines and compounds related through metabolism are used widely in industry. As a result of such applications, they are also dispersed into the environment, thereby creating a potential for human exposure. Four aromatic are known to lead to urinary tract cancer in exposed humans, and some are also responsible for the induction of methemoglobinemia. Some other structurally similar amines are carcinogenic to one or more tissues in laboratory animals. This report summarizes the key information concerning the occurrence, analysis, and toxicology of the aromatic amines and then considers six specific amines in detail.

Aromatic amines are used in dyes, antioxidants, polymers, explosives, pesticides, and pharmacologic agents. N-Hydroxylation of the aromatic amine or N-acetyl derivative, followed by conjugation of the arylhydroxlamine or arylhydroxamic acid, appears to be the key to the activation of aromatic amines. It is not yet possible to predict the potency of a carcinogen in any species. The potency of a carcinogen depends on three factors: the dose of carcinogen required to induce tumors, the time to tumor induction, and the percentage tumor response.

The following substances were studied: Aniline, 4,4'Methylene-Bis (2Chloraniline), 2,4-Diaminotoluene, trifluralin, p-Cresidine, and furazolidone. The following topics are covered: occurrence in the environment, general analysis, metabolism, carcinogenc potency and risk estimation, and epidemiology.

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An Assessment of the Biological and Environmental Effects

Committee on Amines
Board on Toxicology and Environmental Health Hazards
Assembly of Life Sciences
National Research Council

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This report has been reviewed by a group other than the authors according to procedures approved by a Report Review Committee consisting of members of the National Academy of Sciences, the National Academy of Engineering, and the Institute of Medicine.

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EXECUTIVE SUMMARY

Aromatic amines and compounds related through metabolism are used widely in industry. As a result of such applications, they are also dispersed into the environment, thereby creating a potential for human exposure. Four aromatic amines are known to lead to urinary tract cancer in exposed humans, and some are also responsible for the induction of methemoglobinemia. Some other structurally similiar amines are carcinogenic to one or more tissues in laboratory animals.

This report summarizes the key information concerning the occurrence, analysis, and toxicology of the aromatic amines and then considers six specific amines in detail.

HISTORICAL PERSPECTIVE

Aromatic amines comprise one of the major groups of carcinogens. In 1895 Rehn reported that four workmen, three of whom were employed at a single plant manufacturing magenta (fuschin) from crude, commercial aniline, appeared at his clinic with bladder cancer. He correctly deduced that these comparatively rare tumors were associated with the workers' occupation. Hueper (1942) suggested that a limited number of aromatic amines were human bladder carcinogens in humans. Case and his colleagues (1954) confirmed this finding in their classic investigation of the British chemical industry. They demonstrated that 2-naphthylamine, benzidine, and 1-naphthylamine, contaminated with between 4% and 10% of the 2-isomer, were carcinogenic in the human bladder. Melick and his associates (1955, 1971) also showed that 4-aminobiphenyl (xenylamine) was a potent carcinogen in the bladders of those occupationally exposed and of users.

The only other aromatic amine for which there is significant evidence of probable carcinogenicity in humans is 4-ethoxyacetanilide (phenacetin), which was a major component of the analgesics consumed in excessively large quantities by certain well-defined populations. These exposed populations developed renal papillary necrosis and renal pelvic and bladder cancer (Bengsston *et al.*, 1968; Rathert *et al.*, 1973).

Two consequences followed the identification of bladder carcinogens in occupationally exposed humans. First, the manufacture and use of 4-aminobiphenyl and 2-naphthylamine have, for all practical purposes, been phased out, although benzidine continues to be manufactured and used, even in the United States. Traces of a variety of aromatic amines, including 2-naphthylamine, still occur in the environment as the result of combustion of organic materials, including cigarettes. Second, there has been a major research effort to determine which aromatic amines are carcinogenic in laboratory experimental animals (Clayson and Garner, 1976), how they may be measured in the environment, and how their mechanism of action operates (Miller and Miller, 1976). Results from this effort demonstrate that many aromatic amines or their derivatives are indeed potent carcinogens in animals and that they are converted to derivatives of arylhydroxylamines during metabolic activation. These intermediates probably dissociate to electrophilic nitrenium ions which interact with nucleophilic centers in major cell macromolecules (e.g., DNA, RNA, and protein) in exerting their carcinogenic and other toxic effects.

CHARGE TO THE COMMITTEE

The committee was convened by the National Research Council at the request of the Environmental Protection Agency (EPA) to assess the health and environmental effects of amines. The committee decided that the topic could be addressed best by dividing the subject into two parts: (1) aromatic amines and related compounds and (2) aliphatic amines. This report on aromatic amines consists of chapters on the general characteristics of the class followed by chapters concerned with specific chemicals that illustrate problem areas.

The committee reviewed the background information on aromatic amines and related compounds, stressing, for example, general analytic methods, toxicity, mutagenic and carcinogenic properties, and the utility of these substances to industry. It decided that it would exclude chemicals such as 2-naphthylamine, 4-aminobiphenyl and benzidine since they have been extensively and repeatedly reviewed in the past. Benzidine and its cogeners were especially well covered in a recent report by Shriner *et al*. (1978).

Discussions with the EPA led to the selection of the following compounds for intensive review: aniline, 4,4'-methylenebis(2-chloroaniline), 2,4-diaminotoluene, trifluralin and oryzalin, *p*-cresidine, and furazolidone. Both trifluralin and furazolidone are nitro compounds, but were considered relevant to this study because of the relatively easy biological conversion of the nitro groups to the amino group and the fact that arylhydroxylamine derivatives, in which the nitrogen is in an intermediate oxygenated state between the amine and nitro compound forms, is now generally recognized as the proximate biologically active form.

The committee did not address the environmental aspects of exposure after an intensive search of the literature revealed a lack of information on this subject for the compounds selected.

OCCURRENCE, CONTROL, AND ANALYTIC METHODS

Aromatic amines are used in dyes, antioxidants, polymers, explosives, pesticides, and pharmacologic agents. Workers in plants producing these products can be exposed to a health hazard. An evaluation of the data indicating that aromatic amines are potentially toxic in humans indicates that it is prudent to suggest that exposure be held to a minimum both for the worker and others who may be exposed to inadequately controlled wastes associated with manufacture of the products or who are users of the products. Low levels of exposure to aromatic amines may result from the products of destructive distillation of materials containing carbon, hydrogen, and nitrogen, as in smoke from tobacco and the gases resulting from the combustion of fossil fuels. Because of the anticipated changes in the consumption pattern of fossil fuels--those implicit in the promotion of diesel engines and the use of coal--occupational and environmental exposure to the aromatic amines may increase (National Academy of Sciences, 1981). The committee recommends that both the qualitative and quantitative factors that attend these changes be evaluated.

Techniques such as colorimetry, high-pressure liquid chromatography (HPLC), gas chromatography (GC) and mass spectrometry (MS) have been used to analyze aromatic amines. Modification of sampling and clean-up procedures is required for certain substrates, and techniques must be instituted to ensure good recoveries of the more volatile compounds such as aniline. The use of electron-capture derivatives, HPLC, and MS are attractive prospects. Derivatives such as trifluralin and furazolidone require the development of special recovery techniques.

METABOLISM

N-Hydroxylation of the aromatic amine or *N*-acetyl derivative, followed by conjugation of the arylhydroxylamine or arylhydroxamic acid, appears to be the key to the activation of aromatic amines.

Other metabolic reactions, such as acetylation and ring hydroxylation followed by conjugation, are detoxifying pathways.

Understanding of the metabolism of aromatic amines in laboratory animals has advanced to the point that research workers are better able to understand the process in humans. To further this objective, it is first necessary to know which animal most closely approximates the metabolic responses of humans. Assuming that most chemical carcinogens need to be metabolized in the host to active forms in order to exert a carcinogenic effect, it becomes necessary to know how specific carcinogens are activated in animals. If the metabolism of a specific chemical carcinogen in human tissues is qualitatively similiar to that observed in studies of tissues from a susceptible test animal, then a potential carcinogenic effect might also be observed in humans exposed to the chemical. Although the use of humans for testing is difficult or impossible and laboratory animals tests are expensive, it should be feasible to make inferences concerning responses in humans based on *in vitro* tests with human cell lines, mutagenicity testing with human liver S-9 fractions, and careful monitoring of blood and urine of humans accidently exposed to compounds of interest. It would be easier to assess the risk to humans from many aromatic amines if additional biological data on occupationally or otherwise exposed humans were obtained.

STRUCTURE-ACTIVITY RELATIONSHIPS

Many aromatic amine and nitro compounds exhibit the ability to induce cancer in animals. Unless polar groups, such as sulfonic or carboxylic acid substituents, are present in the molecule, these chemicals should be regarded as potentially carcinogenic. Fortunately, however, the most potent carcinogens among the aromatic amines appear to possess certain structural identifying characteristics, such as:

- one, two, or three conjugated aromatic ring systems,
- an aromatic amino group substituted in the position para to the conjugated aromatic system; or
- groups, such as methyl, methoxyl, or fluorine, substituted in relative positions to the amino group.

However, carcinogenicity has been associated with aromatic amines that lack one or more of these characteristics.

A thorough understanding of these relationships may make it possible to predict the potential toxicity of aromatic amines before they are adopted for widespread use. Furthermore, this knowledge and the anticipated increases in the understanding of structure-activity characteristics of aromatic amines and of other chemicals may allow for the selective development of desirable chemical species without the accompanying toxicity.

CARCINOGENIC POTENCY AND RISK ESTIMATION

It is not yet possible to predict the potency of a carcinogen in any species. One prudent approach is to assume that humans are at least as sensitive to these carcinogens as are the most sensitive species. For example, 2-naphthylamine should be assumed to be as potent in humans as it is in dogs.

The potency of a carcinogen depends on three factors: the dose of carcinogen required to induce tumors, the time to tumor induction, and the percentage tumor response. A method for expressing relative potency is described in Chapter 4. Whatever tumor incidence-dose model is used to describe a biologic event, the suggested means of expressing potency values discussed herein may have a considerable advantage in that these values may be derived without excessive effort or data extrapolation. Ways of predicting carcinogenic potency based on the present knowledge of the mechanisms of carcinogenesis urgently need to be improved.

The use of statistical models to estimate possible risk to humans exposed to very low concentrations is filled with uncertainty. Studies of animals usually involve exposure to a high level of a single carcinogen and, sometimes, just one modifying agent. On the other hand, humans are exposed to a wide range of carcinogens and carcinogenesis-modifying agents that may enhance or inhibit the development of cancer especially if the carcinogen exposure is low.

The recently completed ED_{01} experiment conducted by the National Center for Toxicological Research has forced a rethinking of some aspects of dose-response modeling for carcinogenesis. More attention may need to be directed toward the concepts of initiation and promotion and the inherent abilities of chemicals to act in one or the other capacity as well as in both.

EPIDEMIOLOGY

Occupational exposure to 4-aminobiphenyl, 2-naphthylamine, and benzidine has been clearly associated with an increased rate of bladder cancer in workers. The use of 2-naphthylamine-containing ingredients in the British rubber industry was associated with bladder cancer in workers; however, U.S. rubber workers have not exhibited an increased incidence of bladder cancer as a result of exposure to that compound. There is no epidemiologic evidence from which to assess the effects on humans from exposure to the specific chemicals examined in this report.

With the exception of trifluralin, the chemicals assessed in this report have induced cancer at various sites in one or more species of animals. Most toxicologists accept the concept that a demonstration of carcinogenicity in laboratory animals implies that the causative agent is a potential carcinogen in humans. Of the chemicals discussed in this report, 2,4-diaminotoluene (a component of some hair dyes) and 4,4'-methylene-bis(2-chloroaniline)(MOCA) are of most interest because they have been shown to be carcinogenic in animals and because humans are frequently exposed to products containing them. Case-control studies have raised suspicions, while not providing conclusive proof, that the use of widely available hair dyes containing 2,4-diaminotoluene may be associated with cancer of the breast and of other sites. Because of faulty industrial waste disposal methods, many people in Adrian, Michigan have been exposed to MOCA, which is known to be a potent carcinogen in animals. However, there is little evidence upon which to judge its carcinogenicity or other effects to humans.

Retrospective studies are not as likely to provide definitive information on carcinogenicity in humans. In case-control studies of cancer, the recollection of, for example, hair dye use is subject to a high degree of recall bias. In retrospective cohort studies of persons exposed to other chemicals, there is a very imprecise measure of exposure.

The prospective follow-up study is the only realistic study design to evaluate the carcinogenicity of these substances in humans. To determine the effects of hair dyes, it would be necessary to interview women (and possibly men) about their lifetime use of hair dyes and follow them for 5 to 20 years to measure the rate of cancer occurrence. To determine MOCA's health effects, persons exposed occupationally to relatively high levels of MOCA, their families, and preschool children living near the plant would have to be identified, categorized as to level of current (and future) exposure, and followed for 20 to 40 years.

The epidemiologic evaluation of the possible health effects from exposure to low levels of aromatic amines as well as to other substances may be costly and time consuming. To the extent that disease among an exposed group is increased relatively little above background, perhaps less than 50%, the excess may not be detectable against the background variability. One of the best ways to minimize this variability is to conduct prospective follow-up studies, so that the measure of exposure is as precise as possible and does not, for example, depend on memory-based recall of hair dye use. If this strategy is adopted, long term and costly follow-up is the price that must be paid.

ANILINE

In the United States, more than 300 x 10^3 metric tons of aniline is produced per year. It is the parent compound for more than 300 chemical products. A metabolite of aniline, phenylhydroxylamine, is known to cause methemoglobinemia in exposed workers. Although the mechanism is fairly well understood, it now appears that some people are more susceptible to this condition than others. The mechanism of this increased sensitivity needs further investigation. The National Cancer Institute recently reported that exposure to the maximum tolerated dose of aniline led to hemangiosarcomas and other sarcomas of the spleen in rats, but not in mice. Mutagenicity tests produced negative results except in the presence of a comutagen. These findings indicate the need for further research, including carcinogenic studies, possibly on dogs. There is no evidence indicating that aniline causes cancer in humans, but further epidemiologic studies are required. There is also a need to explore the mechanism by which aniline induces splenic tumors in rats and to determine why it is not carcinogenic in mice.

4,4'-METHYLENE-BIS(2-CHLOROANILINE)

Studies in laboratory animals have demonstrated conclusively that 4,4'-methylene-bis(2-chloroaniline) (MOCA) is a carcinogen. Such activity is expected because of the chemical's structural similarity to other carcinogenic aromatic amines. Widespread environmental contamination by MOCA is attributed to one point source in Adrian, Michigan, thus increasing the need for epidemiologic investigations of the exposed population. There is also a need to study the metabolic fate of MOCA so that those who have been highly exposed can be identified by these metabolic indicators and prospective epidemiologic investigations can be facilitated.

2,4-DIAMINOTOLUENE

2,4-Diaminotoluene (2,4-DT) has been used in some hair dye formulations. It is also used as an antioxidant and antiozonant in some rubber products.

When administered orally, 2,4-DT is carcinogenic in rats and mice, leading to liver and mammary gland tumors. This compound is also a potent microbial mutagen, and induces mutations in *Drosophila melanogaster*. There is no information on the mechanism by which 2,4-DT is activated in susceptible species, including rats and mice. Given the positive demonstration of carcinogenicity in two animal species and the data on the genotoxic effects of 2,4-DT in *in vitro* systems, it is prudent to assume that humans may be under some increased risk from exposure to 2,4-DT.

TRIFLURALIN

At the request of the Environmental Protection Agency, this report assesses only the potential mutagenic and genotoxic properties of trifluralin, a widely used herbicide. During its commercial preparation, an impurity, dipropylnitrosamine(NDPA), is produced. Since NDPA is a mutagen, it is difficult to assess this property in trifluralin. Most mutagenicity studies of trifluralin have produced negative results. Those that are positive for chromosomal damage and aneuploidy may be due to the presence of NDPA. Parallel studies with NDPA have not been reported. NDPA-free trifluralin and pure NDPA need to be tested, in tandem, for their abilities to induce chromosomal damage and aneuploidy. There is an inadequate data base from which to evaluate the potential hazards of trifluralin and NDPA, to DNA and cell spindles in laboratory animals or humans. Should NDPA be a germinal mutagen, exposures are expected to occur at such low levels that its mutagenic potential should be correspondingly low. Current manufacturing practice has considerably reduced the level of NDPA in trifluralin and its formulations.

Positive mutagenicity test results obtained with trifluralin have subsequently been attributed to a 177-ppm NDPA impurity contained in the trifluralin rather than to the test chemical itself. There is a need for additional mutagenicity testing on NDPA-free trifluralin for comparison with the existing studies. Until these studies are performed, pure (NDPA-free) trifluralin should not be considered mutagenic.

p-CRESIDINE

p-Cresidine is used almost exclusively as a chemical intermediate in the manufacture of dyes. Other than a cancer bioassay conducted by the National Cancer Institute, there is virtually no other existing biologic data on this compound. Chronic oral exposures to p-cresidine produced bladder cancer in both male and female rats and mice as well as hepatocelluar carcinomas in male rats and female mice. In a preliminary investigation, the compound showed dose-response mutagenicity without metabolic activation in the *Salmonella* assay.

Because of the lack of data, it is difficult to evaluate the potential health effects of this compound. Nonetheless, it must be considered as a potential carcinogen in humans on the basis of the carcinogenicity demonstrated in rats and mice. The preliminary mutagenicity data appear to show a positive response for p-cresidine, but confirmation is needed. Additional data are also needed on metabolism, metabolic activation, mutagenicity, and genetic toxicity in both animal and human *in vitro* test systems.

FURAZOLIDONE

Furazolidone is one of the 5-nitrofurans currently approved for use as a systemic veterinary medicine in the United States, thereby finding its way into some edible tissues. It has also been used to treat bacillary dysentery, typhoid, and other infectious diseases in

4.0 ppb with current analytical methods. Various spectrophotometric and thin layer chromotography procedures have failed to yield satisfactory and reproducible recoveries at these levels.

Furazolidine has exhibited carcinogenic effects in male and female rats and mice just as other 5-nitrofurans have done. A variety of different tissues in each species have displayed these effects. The compound is highly mutagenic in both microbial (Escherichia coli) and insect (Drosophila melanogaster) test systems, produces chromosomal damage (breakage, sister-chromatid exchange, mitotic suppression) in human lymphocytes, and forms interstrand cross-linking in bacterial (Vibrio cholera) DNA.

For the reasons discussed above, the use of furazolidone is now being reviewed by the Food and Drug Administration. Resolution of this matter awaits the development of a sufficiently sensitive and reliable analytical method. Any identified problem most certainly would be associated with the veterinary use of furazolidone. A "solution" may involve the substitution of an efficacious product known not to have the mutagenic and carcinogenic potential of furazolidone.

RESEARCH RECOMMENDATIONS

The following recommendations have been excerpted from the various chapters to highlight and focus attention on specific topics the committee felt deserved further consideration.

Occurrence in the Environment

The anticipated changes in the use of fossil fuels, implicit in the promotion of diesel engines and coal, may raise existing environmental levels of some aromatic amines. Accordingly, it is recommended that steps be taken to evaluate both the qualitative and quantitative factors that attend these changes in practice.

General Analysis

Techniques must be developed to analyze for the presence of the more volatile compounds such as aniline. Furthermore, methods must be developed to determine the kinds and amounts of metabolites present in persons exposed to these compounds.

Metabolism

Understanding of the metabolism of aromatic amines in laboratory animals has advanced to the point that it can be extended to improve the understanding of this process in humans. First it is necessary to know which animal most closely approximates the metabolic responses of humans. Assuming that most chemical carcinogens need to be metabolized in the host to active forms in order to exert a carcinogenic effect, it becomes necessary to know how specific carcinogens are activated in animals. If the metabolism of a specific chemical carcinogen in human tissues is qualitatively similiar to that observed in studies of tissues from a susceptible test animal, then a potential carcinogenic effect might also be observed in humans exposed to the chemical. Although the use of humans for testing is difficult or impossible, it should be feasible to make inferences based on in vitro tests with human cell lines, mutagenicity testing with human liver S-9 fractions, and careful monitoring of blood and urine of humans accidently exposed to compounds of interest. It would be easier to assess the potential risk to humans from many aromatic amines if additional biological data on humans were obtained.

Carcinogenic Potency and Risk Estimation

More attention needs to be focused on the concepts of initiation and promotion and the inherent abilities of chemicals to act in one or the other capacity as well as in both. Also, ways of predicting carcinogenic potency based on the present knowledge of the mechanisms of carcinogenesis urgently need to be expanded. In the absence of contrary evidence, aromatic amines which lack polar groups should be regarded as carcinogens.

Epidemiology

Epidemiologic methodology needs to be improved so small differences between exposed and control groups can be detected above the background variability. One of the best ways to minimize this variability is to conduct prospective follow-up studies so that the measure of exposure is at least as precise as possible. Of all the compounds discussed in this report, the widespread exposure to 4,4'-methylene-bis(2-chloroaniline) (MOCA) represents the best opportunity for this approach.

Aniline

Although there has been considerable research on aniline, there is still much to be learned about its possible health effects. A compond of such industrial importance deserves to be studied more thoroughly. The hemangiosarcomas and sarcomas of the spleen and other organs observed at the maximally tolerated dose (MTD) in the National Cancer Institute bioassay need to be examined further in another lifetime feeding study at three or four dose levels in a different strain of rat to interpret the significance of previous observations.

A carcinogenicity study using Syrian golden hamsters may also be useful since these animals to develop bladder tumors after exposure to other aromatic amines. In addition, a long term (preferably 8-10 years) dog-feeding study at the MTD should allay any suspicions concerning the possible role of aniline in the causation of human bladder cancer. The only test on dogs was conducted many years ago on a few animals for too short a duration. Further studies on the metabolites of aniline in urine should be directed toward explaining the failure of this compound to induce bladder cancer in dogs (if this failure is confirmed). Special attention should be paid to N-hydroxylated urinary metabolites. Moreover, studies should be conducted on its potential for teratogenicity and reproductive toxicity.

Epidemiological investigations on workers exposed to aniline should also be undertaken. Monitoring of urine for aniline metabolites to confirm and quantitate exposures should be considered.

4,4'-Methylene-bis(2-chloroaniline) (MOCA)

Individuals exposed to MOCA in Adrian, Michigan as a result of faulty industrial waste methods and others exposed to the compound should be studied further to learn whether or not the compound is carcinogenic in humans. First, the metabolic disposition of MOCA should be explored so that methods for evaluating exposure can be developed. Then these methods should be applied, to the population at risk, including an evaluation of necropsy specimens from any member of this population who dies during the course of this investigation. Such studies would clarify the potential risk to individuals, and aid in monitoring the effects of the cleanup efforts. The final step is the prospective surveillance of this population to determine whether exposure to MOCA increases their tendency to develop cancer. In addition, studies on the potential for teratogenicity and reproductive toxicity should be conducted.

2,4-Diaminotoluene (2,4-DT)

Recommendations for future research on 2,4-DT include studies of the mechanism by which the chemical is activated in rats, mice, and humans, testing for carcinogenicity in other species to obtain more data on the relationship between metabolism and carcinogenicity, and examination of the *in vitro* metabolism of 2,4-DT in human tissues. The resulting data would facilitate estimatation of risk to humans exposed to 2,4-DT. Studies on the potential for teratogenicity and reproductive toxicity should also be conducted.

Trifluralin

Tandem mutagenicity studies on pure trifluralin and its contaminant, N-nitrosodipropylamine should be conducted to determine which compound is responsible for chromosomal damage and aneuploidy noted in previous studies.

p-Cresidine

In vitro studies of human tissues and tests with animals should be conducted to gather data on the metabolism, metabolic activation, mutagenicity, and genetic toxicity of p-cresidine. Furthermore, studies on the potential for teratogenicity and reproductive toxicity need to be performed.

Furazolidone

A sufficiently sensitive and reliable analytical method for furazolidone is needed. Moreover, studies on that compound's potential for teratogenicity and reproductive toxicity should be conducted.

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Chapter 1

CONTROL, OCCURRENCE, AND IDENTIFICATION

Although concern for the health effects of aromatic amines has focused primarily on industrial use of these substances, it is becoming increasingly evident that there are many sources of exposure to these compounds and to their precursors. The facile biochemical reduction of arylnitro compounds by both mammalian and microbial organisms necessitates that their precursors be identified so that the distribution of potentially hazardous aromatic amines can be surveyed. Similarly, azo dyes are readily reduced to free amines by a variety of enzymes. Given the metabolic capacities likely to be involved, it is prudent to regard any N-substituted aromatic compound as a potential aromatic amine.

In general, aromatic amine derivatives to which humans might be exposed are either synthesized intentionally for some specific commercial use or produced by enzymic reduction of aromatic nitro or azo compounds or are formed inadvertently as byproducts in processes apparently directly or indirectly related to combustion.

Commercial Products

The development of the synthetic dye industry in Europe during the latter half of the 19th century led to the first recognition of arylamine-induced bladder cancer in humans. Since that time, industrial organic chemistry has become more sophisticated and it is not surprising that aromatic amines are now widely marketed in dyes and as compounds to be used in various manufacturing processes. The synthesis of these substances can cause occupational health hazards, and inadequate control of wastes associated with their production can result in contamination of the environment. Furthermore, the practical use of the products can expose both workers and consumers to their dangers.

Because of the various uses intended for the aromatic amines and the ingenuity of the chemist, commercial products have various and ever-changing compositions. Amines are integral to the following compounds:

- dyes
- antioxidants
- polymers
- explosives
- pesticides
- pharmacologic agents

Byproducts and Combustion Processes

In contrast to the often large-scale industrial synthesis, aromatic amines are also produced inadvertently in low concentrations as byproducts of processes that expose organic materials to elevated atmospheric temperatures. The combustion of organic materials can generate aromatic amine derivatives by two different mechanisms. The partial combustion or pyrolysis of nitrogen-containing organic material can produce both azoheterocyclics and arylamine compounds, as exemplified by the detection of 22 µg of 2-naphthylamine in the smoke of 100 cigarettes (Hoffmann and Wynder, 1976), amino-substituted carbolines in amino acid pyrolysates (Kosuge et al., 1978), and indirect mutagens believed to be primary aromatic amines in synthetic fuels (Epler et al., 1980).

The presentation by Guerin (1980) and subsequent discussion by several authors agreed that most of the observed mutagenic activity resulting from many different samples derived from coal, shale-derived oil, and petroleum crudes, could be attributed to an alkaline isolate fraction constituting only a fraction of a percent of the sample mass. These samples contained (among other things) identifiable polynuclear aromatic amines.

A second, more indirect, mechanism that produces amines is seen in the formation of nitroaromatic compounds as a consequence of combustion processes. Polycyclic aromatic hydrocarbons can be nitrated by nitrogen oxides formed at high temperatures from atmospheric nitrogen. These compounds may be formed during or immediately after the combustion process, but the possibility of their subsequent photochemical formation has not been excluded. Indirect evidence for the formation of nitrated aromatics as byproducts of the combustion process comes from two sources. The model studies of Pitts and his collaborators (1980) demonstrated that polycyclic aromatic hydrocarbons were readily nitrated by levels of nitrogen oxides found in the atmosphere. The second line of evidence comes from analysis of the mutagenicity of particulates collected from internal combustion engines (Claxton and Huisingh, 1980) and from the atmosphere (Wang *et al*., 1980). The mutagenicity of these materials in *Salmonella typhimurium* are decreased if tested in strains that are deficient in the ability to be reverted to prototrophy by arylnitro compounds.

Thus, the population may be exposed to arylamine precursors from several noncommercial sources (Lofroth, 1978):

- tobacco smoke,
- food pyrolysates,
- synthetic fuels,
- internal combustion engines,
- atmospheric particulates, and
- fossil fuel-fired power plants.

The last three sources contain direct-acting mutagens to bacteria that contain nitroreductase which suggests the presence of nitroaromatics.

Unlike the long-recognized potential hazards of the industrially produced aromatic amines, the widespread distribution of these compounds in prepared food and the environment has only recently been demonstrated. Thus, the analytic methodology required for their study is only now being developed. When the appropriate techniques are available, it will be possible to evaluate the compounds involved, the levels of exposure, and the biologic consequences of contamination.

RECOMMENDATION

The anticipated changes in the use of fossil fuel, implicit in the promotion of diesel engines and coal, may raise existing environmental levels of aromatic amines. Accordingly, the committee recommends that steps be taken to evaluate both the qualitative and quantitative factors that attend these changes in practice.

GENERAL ANALYTICAL PROCEDURES

Aniline, p-cresidine, 2,4-diaminotoluene, and MOCA [4,4'-methylene-bis(2-chloroaniline)] are primary aromatic amines and should respond to the following general analytic procedures. Trifluralin, a tertiary aromatic amine, and furazolidone, a nitrofuran, do not respond to these procedures and must be analyzed by other means as discussed at the end of this chapter.

General Procedures for Primary Aromatic Amines

Colorimetric Methods. A spectrophotometric method for amines, amino acids, and a peptide using 2,4,6-trinitrobenzenesulfonic acid (TNBS) as the chromogenic agent was reported by Satake et al. (1960). Rinde and Troll (1975) modified this method to determine free benzidine in the urine from monkeys dosed with a benzidine-based dye. Urine samples were extracted with chloroform and back-extracted with 0.01 M hydrochloric acid. The acid extract (pH 5) was reacted with TNBS and the yellow color extracted into chloroform for analysis. Extraction of the reaction products into an organic solvent was required to prevent excess reagent in the aqueous phase from interfering with the analysis. TNBS was also used as a spray to render the spots visible on thin-layer chromatographic (TLC) plates. Although the procedure is sensitive in the nanomole range, it lacks the specificity usually required of analytic methods. Nevertheless, the procedure is currently used by the National Institute for Occupational Safety and Health (NIOSH) to monitor urine from industrial workers (Lowry _et al._, in press).

The reagent fluorescamine (Fluram) was introduced by Udenfriend _et al._ (1972) to quantify aliphatic amines in a sensitive fluorescent assay. Aromatic amines form fluorescent products just as the aliphatics do, but the aromatic products are unstable. However, the parent compounds do form stable yellow derivatives. Rinde and Troll (1976) used Fluram to develop a colorimetric procedure to identify several carcinogenic aromatic amines. Dry residues of the amines were reacted with 50 μl of Fluram solution (1 mg/ml in glacial acetic acid) for 10 minutes. The reaction was stopped by adding 0.5 ml of methanol. Optical density readings of the yellow color were made in 0.5 ml cuvettes in a spectrophotometer set at the approximate wavelength for maximum absorption. Fluram is said to react only with aromatic amines in a glacial acetic acid medium. The reaction can be performed directly on TLC plates to add specificity to the measurement, and the yellow product can be quantitatively eluted from the TLC plates. Fluram is colorless, so blanks appear as zero, a distinct advantage over procedures employing a TNBS reagent. The sensitivity of the procedure ranges from 2 to 10 nanomoles for compounds such as benzidine and

2-naphthylamine.

Spot Tests. Strict procedural guidelines imposed by the Occupational Safety and Health Administration (OSHA) concerning 14 potentially carcinogenic chemicals (including some aromatic amines) prompted Weeks *et al*. (1976) to investigate various spot-test procedures for the compounds. Chromogenic agents, such as Ehrlich's reagent, *p*-diethylaminobenzaldehyde, *p*-dimethylaminocinnamaldehyde, chloranil, and chloramine T, and fluorogenic agents, such as Fluram and isomeric phthaldehydes, were evaluated. The limit of detection values in terms of grams of analyte per square centimeter of surface being examined ranged from the low nanogram to the 5-μg level, depending on the compound, sampling technique, and surface involved.

Thin-Layer Chromatography. There are numerous procedures for separating and detecting aromatic amines. In recent years, reaction chromatography has been found to identify amines most successfully. In this technique, a derivative is prepared, and then both the derivative and the parent compound are subjected to separation. Some of the derivatives of aromatic amines that have been prepared for this purpose are 3,5-dinitrobenzamides, 4-dimethylaminobenzeneazo-4-benzamides, 2,4-dinitrophenylamines, *p*-toluenesulfonamides, and arylazo-2-naphthols. Isomeric toluidines and aniline, which are difficult to separate as salts, can be converted into bromine derivatives; dansyl chloride has also been used. More recently, Franc and Koudelkova (1979) investigated TLC separations of 128 differently substituted aromatic amines and their 2,4-dinitrophenyl derivatives. Three solvent systems were used and in certain cases, assays were completed by paper electrophoresis. The derivatives were prepared by reacting the amines with 1-fluoro-2,4-dinitrobenzene. The three-solvent system used with Silufol UV-254 were diethyl ether-cyclohexane (4:3) and ethyl acetate-N-propanol-ammonia (5:4:1) and (2:1:2). The aromatic amines were detected by spraying with a 1% solution of Ehrlich's reagent in 1 M hydrochloric acid. The derivatives were detected by spraying with 5% stannous chloride to reduce the nitro groups to amino groups. After the chromatogram dried, Ehrlich's reagent was used.

Lepri et al. (1978) in experiments with soap TLC studied the behavior of 35 primary aromatic amines on layers of silanized silica gel alone or impregnated with 2% or 4% triethanolamine dodecylbenzenesulfonate solutions. Eluents such as a mixture of 1 M acetic acid and 30% methanol were employed. Ehrlich's reagent was used to detect the amines. Several separations that could not be made either by ion exchange or reverse-phase chromatography were carried out.

Additonal TLC procedures for specific compounds are discussed later in this chapter.

Gas Chromatography. Many of the aromatic amines are amenable to the conditions imposed by gas chromatography (GC) and can be measured by using the nonspecific flame ionization detector (FID), where extremely high sensitivity (e.g., low nanogram or picogram range) is not required. Also, the rubidium-sensitized thermionic-type nitrogen/phosphorus (N/P) detector responds to the compounds with various degrees of sensitivity and specificity. Nevertheless, most of the work in GC methods development for the aromatic amines has centered around the formation of halogenated derivatives with subsequent analysis by electron-capture (EC) GC. Such procedures greatly enhance sensitivity and often improve stability and GC characteristics.

Francis _et_ _al_. (1978) demonstrated that flophemesyl (pentafluorophenyl-dimethylsilyl) derivatives of amines and other classes of organic compounds could be prepared and analyzed with high sensitivity by EC-GC. The amine (10 mg or less) dissolved in toluene (30 μl) was reacted with 30 μl of flophemesylamine in a sealed vial at 60°C for approximately 15 minutes. Aniline and 2-phenylethylamine were among the amines evaluated. The gas chromatography was accomplished by using a 1.5-m glass column packed with 10% SE-30 on Chromosorb P AW DMCS and a ^{63}Ni EC detector; sensitivities were reported in the picogram range.

Nony and Bowman (1978, 1980) adapted the method of Walle and Ehrsson (1970) to assay several aromatic amines as their pentafluoropropionyl (PFP) or heptafluorobutyryl (HFB) derivatives. The amine (10 μg or less) in 1.5 ml of benzene is added to a tube containing 0.5 ml of trimethylamine catalyst (0.05 M in benzene), then 50 μl of heptofluorobutyric acid anhydride is added. The sealed system is heated at 50°C for 20 minutes, the reaction is terminated, and the benzene phase is extracted by using 2 ml of phosphate buffer (pH 6). The benzene phase, dried over sodium sulfate, is analyzed on a 1.8-M glass column packed with 5% Dexsil

300 on Gas Chrom Q by EC-GC employing a ^{63}Ni detector. Sensitivities are generally in the low picogram range; however, the procedure requires modification to preclude low recoveries of volatile compounds such as aniline. No cleanup procedures were used in the assays. In another study using PFP derivatives, Bowman and Rushing (1977) used both EC and N/P detectors to assay for trace levels of 3,3'-dichlorobenzidine in animal feed, wastewater, and human urine. The use of cleanup procedures using XAD-2, silica gel, and/or liquid-liquid partitioning permitted the detection of low ppb levels of the compound in feed and low ppt levels in wastewater and human urine. These procedures could probably be adapted to assay all four primary aromatic amines discussed in this report.

High Pressure Liquid Chromatography. Although few high-pressure liquid chromatography (HPLC) methods for aromatic amines have been described in the literature, the technique appears promising when the compounds are not amenable to GC or derivatization techniques or when optimum sensitivities are not required. Popl et al. (1978) measured retention data for polar-substituted benzenes and naphthalenes, including several aromatic amines, by using reverse-phase, liquid-liquid chromatography on macroporous polystyrene gel with methanol-water and acetonitrile-water as the eluents. Riggin and Howard (1979) employed electrochemical detection (thin-layer glassy carbon electrode) for HPLC assays of benzidine and related compounds at ppb levels. Rice and Kissinger (1979) described a specific method using HPLC with amperometric detection (carbon paste electrode vs silver/silver chloride reference electrode) for benzidine and its mono- and diacetyl metabolites in human urine. A detection limit of 10 ppb based on twice noise was reported for benzidine. Nony and Bowman (1980) reported HPLC methods for a variety of aromatic amines and related compounds in hamster and human urine using a Bondapak C_{18} column, mixtures of methanol-phosphate buffer (pH 6) as the mobile phase, and a variable ultravoilet absorption detector set at the appropriate wavelength for maximum response. The detection limit for benzidine in the urine by this procedure was approximately 180 ppb, based on twice background.

Where applicable, the use of a fluorescence detector generally enhances the sensitivity and specificity of assays by HPLC.

Other Aromatic Amines

Considerable research has been conducted on methodologies for detecting trifluralin because of its widespread use as a herbicide. Because the compound captures electrons well and is amenable to GC, the EC-GC procedures (with minor modifications) should meet most of the requirements. Alternative methods include TLC, HPLC, and mass spectrometry (MS).

There are adequate procedures (e.g., colorimetry, TLC, and HPLC) for analyzing relatively high concentrations of furazolidone. However, assays for the compound at low ppb levels in various substrates appear almost intractable. Although HPLC is probably the best procedure available, deficiencies in cleanup prior to injection and in the inherent sensitivity limits of the system may preclude its use at low ppb levels. Research in the areas of sample cleanup and mass spectrometry methodology is the next step to improve the stateoftheart.

CONCLUSIONS

Techniques such as colorimetry, TLC, GC, HPLC, and MS have been used to analyze primary aromatic amines. Modifications of sampling and cleanup procedures may be required for different substrates, and techniques must be instituted to ensure adequate measurements of the more volatile compounds, such as aniline. Nevertheless, existing procedures with appropriate modifications should satisfy most analytic requirements. The use of EC derivatives, HPLC, and MS are attractive prospects.

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Chapter 2

METABOLISM OF AROMATIC AMINES

The presence of an amine group has a strong tendency to activate the aromatic ring, resulting in a complex pattern of metabolism and a multiplicity of metabolites. For more than 40 years, it was assumed that 2-naphthylamine was simply metabolized to 2-amino-1-naphthylsulfate in dogs (Wiley, 1938). Subsequent studies with ^{14}C- labeled 2-naphthylamine indicated that although 90% of the radioactivity could be accounted for by this metabolite, seven other metabolites were also present. In addition, metabolism in the dog is much simpler than in other species. For example, acetyl metabolites are not formed in dogs.

These results illustrate the difficulty of conducting studies of metabolism to delineate the best test species for evaluating a new compound when there is little or no information concerning the identity of the metabolite responsible for toxic or pharmacologic effects. The active metabolite could well be quantitatively insignificant. In addition, despite the difference in acetylating ability between humans and dogs, the dog does appear to be a good test species for induction of bladder cancer because it develops bladder cancer from the same amines that humans do, which is a more important consideration (Radomski, 1979b). However, the high cost of maintenance and long lifespan of dogs may preclude their routine use.

Both aromatic amines and amides are extensively metabolized by enzyme systems, principally located in the liver. These enzyme systems are usually divided into two groups: phase I and phase II (Williams, 1959). During phase I metabolism, one or more polar groups (such as hydroxyl) are introduced into the hydrophobic parent molecule, thus allowing a "handle" or position for the phase II conjugating enzymes (such as uridine diphosphoglucuronyl transferase) to attack. The conjugated products are sufficiently polar that these "detoxified" chemicals can be more efficiently excreted from the cell and from the body.

One of the more interesting phase I enzyme systems is a group of enzymes known collectively as the cytochrome P-450-mediated monooxygenases (Cooper *et al*., 1975; Gillette *et al*., 1972; Haugen *et al*., 1975; Johnson, 1979; Lu and Levin, 1974; Neims *et al*., 1976; Thorgeirsson and Nebert, 1977). This enzyme system is involved in a wide range of biologic activities, as it mediates the metabolism of numerous, structurally diverse substrates. It catalyzes the metabolism of many drugs, the transformation of steroids, cholesterol, and bile acids, and the activation and detoxification of a large number of carcinogens. Among the carcinogens extensively metabolized by the cytochrome P-450-mediated monooxygenases are the aromatic amines and amides. This metabolism involves both activation (e.g., *N*-hydroxylation) and detoxification (e.g., C-hydroxylation). Therefore, a balance exists in each tissue between the enzymes that potentiate and those that detoxify xenobiotics. This balance is known to vary with species, sex, age, tissue, hormonal status, and exposure of the animal to certain xenobiotics (Cooper *et al.*, 1974; Gillette *et al.*, 1972; Lu and Levin, 1974; Neims *et al.*, 1976; Thorgeirsson and Nebert, 1974).

The terminal oxidase of these monooxygenase systems is a group of hemeproteins collectively termed cytochrome P-450. The cytochromes are characterized by their spectral absorption maximum, which occurs at 450 nm when they are associated with carbon monoxide in their reduced state. Recent efforts have successfully resolved and characterized multiple forms of the cytochrome, and the study of these isolated forms of cytochrome P-450 provides information regarding the properties of individual cytochromes and aids investigations concerned with regulating the occurrence of each cytochrome.

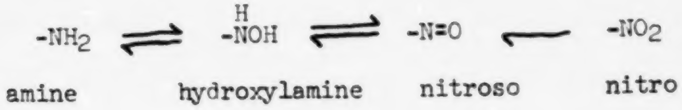
The term "multiple forms of cytochrome P-450" refers to experimentally distinguishable forms of the cytochrome that occur naturally in a single species. The significance of multiplicity to the many biologic processes in which the cytochrome has been implicated will depend largely on the difference in regulatory and functional properties of the individual forms. The most extensively purified and characterized forms of the cytochromes have been those that are induced by compounds such as phenobarbital or the group of compounds designated as arylhydrocarbon(Ah)-inducers which include 3-methylcholanthrene, β-naphthoflavone, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

The role of the different forms of cytochrome P-450 in the metabolic processing of aromatic amines and amides has not been extensively studied. The existing data, however, indicate that the first step in the metabolic activation of this class of chemicals, namely *N*-hydroxylation, may be, at least in some species, catalyzed by a single form of cytochrome P-450. The clearest demonstration of this selectivity in the oxidative metabolism of aromatic amines and amides involves the metabolism of 2-acetylaminofluorene (AAF) by four purified forms of rabbit cytochrome P-450 (Johnson *et al*., 1980). Of these four forms, two (forms 3 and 6) are exclusively involved in C-hydroxylation (i.e., detoxification), one (form 4) is solely responsible the *N*-hydroxylation (i.e., metabolic activation), and one (form 2) does not catalyze either C- or *N*-hydroxylation of the AAF molecule. Also, although the evidence is indirect, genetic studies in mice on the regulation of *N*-hydroxylation of AAF indicate that one or very few genes are responsible for the induction of the cytochrome P-450 form that catalyzes *N*-hydroxylation of AAF (Thorgeirsson *et al*., 1977).

The occurrence of each cytochrome is dependent on many factors, and the relative role of each cytochrome must be integrated with other processes occurring during metabolism and carcinogenesis. Thus, it is difficult to predict the effect of these metabolic differences on carcinogenesis. Despite these uncertainties, metabolism of aromatic amines and amides plays an important role in understanding the carcinogenic potential of these compounds by either lifetime animal or short-term *in-vitro* tests. The outcome of this testing has major consequences for health, society, and the economy. Thus, there is a need for a more clearly defined relationship between the elements of metabolism of aromatic amines and carcinogenicity.

OXIDATION

The primary metabolic attack on aromatic amines is usually oxidation. Two types of oxidation may occur: oxidation of the nitrogen atom (N-oxidation) and oxidation of carbon of the aromatic ring (C-oxidation). Primary aromatic amines may be oxidized through the following stages:



$$\underset{\text{amine}}{-NH_2} \rightleftharpoons \underset{\text{hydroxylamine}}{-\overset{H}{N}OH} \rightleftharpoons \underset{\text{nitroso}}{-N{=}O} \leftarrow \underset{\text{nitro}}{-NO_2}$$

There is little evidence that aromatic amines are oxidized to nitro compounds. On the other hand, the nitro compound is reducible through all the above steps to the amine. Secondary (N-alkyl aromatic amines) and tertiary amines are also N-oxidized. Tertiary amines are oxidized to the N-oxide only. Tertiary amines may also be dealkylated to secondary amines (e.g., dimethylaminoazobenzene to monomethylaminoazobenzene). Secondary amines may be partially N-dealkylated with the formation of hydroxylamines (Zeigler *et al*., 1969). Acetamides are N-hydroxylated with the formation of hydroxamic acids (Miller *et al*., 1960). Hydroxamic acids are quite stable, in contrast to the notorious instability of arylhydroxylamines. At present, arylhydroxylamines are believed to be the proximal carcinogens in the induction of bladder cancer by some aromatic amines. The esters of hydroxamic acids are believed to be the proximal carcinogenic metabolites responsible for the induction of liver cancer by the acetamides (Radomski, 1979a).

Activation of the free amine group of an aromatic amine results in the metabolic hydroxylation of the aromatic ring (C-hydroxylation). The positions attacked generally correspond to the regions of highest electron density (Weisburger and Weisburger, 1958). Thus, aniline and 1-naphthylamine are primarily hydroxylated in the 3 position and secondarily in the 2 position. 2-Naphthylamine and 4-aminobiphenyl, on the other hand, are primarily hydroxylated in the 1 position and the 3 position, respectively (Radomski, 1979a). 2-Aminofluorene either has been more extensively studied or more positions on the aromatic nucleus are fairly equivalent in electronic density, since metabolites hydroxylated in the 1, 3, 5, 7, 8, and recently the 9 positions have been detected (Weisburger and Weisburger, 1958).

GLUCURONIDATION

Perhaps the most important detoxification process is conjugation of metabolites of aromatic amines with glucuronic acid. As far as is known, all species are capable of this metabolic reaction. This conjugation is generally regarded as a phase II process in which the hydroxyl groups introduced by the liver mixed -function oxidase system in phase I are further modified. Conjugation with glucuronic acid results in highly polar metabolites that are rapidly excreted by the kidney through filtration without reabsorption or sometimes through active secretion. Glucuronides are also excreted in the bile (Mandel, 1971).

The conjugation does not occur directly with glucuronic acid but requires an activated intermediate, uridine diphosphoglucuronic acid (UDPGA). The reaction is catalyzed by the enzyme glucuronyl transferase, which occurs in liver microsomes according to the following scheme (Mandel, 1971).

$$\text{glucose-1-phosphate} + \text{UTP} \xrightarrow{\text{pyrophosphorylase}} \text{UDP-glucose} + \text{pyrophosphate}$$

$$\text{UDP-glucose} + 2\text{NAD}^+ + H_2O \xrightarrow[\text{dehydrogenase}]{\text{UDPG-}} \text{UDP-glucuronic acid} + 2\text{NADH} + 2H^+$$

$$\text{UDP-glucuronic acid} + \text{Ar-OH} \xrightarrow[\text{transferase}]{\text{glucuronyl}} \text{Ar-O-glucuronic acid} + \text{UDP}$$

Ar-OH = hydroxylated aromatic amine

UTP = uridine triphosphate

Glucuronidation occurs primarily on hydroxyl groups, but may also occur with carboxyl and amine groups. The formation of highly acidic, labile N-glucuronides may or may not be enzymatic. In a newly discovered metabolic reaction, hydroxylamines formed by the N-hydroxylation of aromatic amines are conjugated with glucuronic acid with the formation of an N-C conjugate (Kadlubar *et al*., 1977; Radomski *et al*., 1973, 1977). For the aromatic amines that induce bladder cancer, these conjugates may represent the carrier form, which transports the carcinogenic metabolite (hydroxylamine) as an aglycone from the site of N-hydroxylation in the liver to the bladder. In the bladder, the acid pH of the urine and/or the presence of β-glucuronidase in the mucosa liberates the hydroxylamine to produce its carcinogenic action (Radomski _et_ _al_., 1977).

SULFATION

A perhaps less important phase II synthetic mechanism carried out by all species of test animals is conjugation of hydroxyl groups with sulfuric acid (sulfate). Thus, the phenolic hydroxyl groups introduced on the aromatic nuclei (phase I) are used to increase the polarity of the original, relatively hydrophobic amine. The primary amine groups may also be directly conjugated, producing _N_-sulfate conjugates (sulfamates) that are readily hydrolyzed in weakly acidic solutions and by ubiquitous hydrolytic enzymes (sulfatases). Of course, these enzymes also hydrolyze O-sulfates. As with the formation of glucuronides, sulfate (SO_4-) must first be activated according to the following scheme (Mandel, 1971).

$$SO_4^- + \text{ATP} \xrightarrow[\text{sulfurylase}]{\text{ATP-}} \text{adenosine-5'-phosphosulfate(APS)} + \text{pyrophosphate}$$

$$\text{APS} + \text{ATP} \xrightarrow[\text{kinase}]{\text{APS-phospho-}} \text{3'-phosphoadenosine-5'-phosphosulfate(PAPS)} + \text{ADP}$$

$$\text{PAPS} + \text{RZH} \xrightarrow[\text{transferase}]{\text{sulfo-}} \text{R-Z-}SO_3H + \text{3-phosphoadenosine-5'-phosphate}$$

where Z is O or NH

The key enzymes in the process are sulfotranferases (sulfokinases). Several distinct enzymes that exhibit considerable substrate specificity are known to exist.

Because the total sulfate pool is quite limited, it is readily exhausted when large amounts of exogenous chemicals are administered. Thus, sulfate conjugation becomes quantitatively less important with increasing doses.

Conjugation of hydroxamic acids with sulfate may also occur. This reaction has been postulated as being responsible for the final activation of N-hydroxy-N,2-fluorenylacetamine in the induction of liver cancer (Irving, 1979).

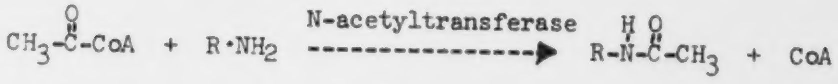
ACETYLATION

Primary amines are acetylated to an appreciable extent by many animal species. Secondary and tertiary amines are never acetylated (Mandel, 1971). The metabolic reaction apparently occurs in the reticuloendothelial cells of the liver, but not in the parenchymal cells (Govier, 1965). Mucosa of the spleen and intestines are also capable of acetylation (Mandel, 1971). For exogenous compounds, acetylation occurs because of the presence in tissues of acetyl-coenzyme A, a prominent component in the Krebs cycle.

Most species also contain a hydrolytic enzyme, a deacetylase, which is capable of removing the acetyl group from acetamides. However, the ability to deacetylate is apparently not absolute; trace amounts of 2-naphthylamine were detected in the urine of a man who had ingested 2-naphthylacetamide (Conzelman, personal communication). The failure of the dog to acetylate aromatic amines

(Marshal, 1954) may be due to the dog's powerful deacetylating ability, the deficiency of an enzyme, or the presence of an inhibitor (Leiberman and Anaclerio, 1962). In the rat, aromatic amines and their acetylated products appear to exist in biologic equilibra with each other (Krebs _et al_., 1947; Peters and Gutmann, 1955). This finding is also evident from the observation that 2-aminofluorene and _N_,2-fluorenylacetamide yield very similar patterns of acetylated and nonacetylated metabolites.

Almost all metabolic alterations of exogenous compounds result in increased polarity of the metabolized compounds. This property is very important to survival: in this manner, the animal is able to dispose of potentially noxious substances efficiently. Acetylation, however, usually results in compounds having decreased polarity. Acetylation of aromatic amines is carried out with the aid of the enzyme _N_-acetyltransferase, according to the following scheme:



$$CH_3-\overset{\overset{O}{\|}}{C}-CoA \;+\; R\cdot NH_2 \xrightarrow{\text{N-acetyltransferase}} R-\overset{H}{N}-\overset{\overset{O}{\|}}{C}-CH_3 \;+\; CoA$$

_N_-acetyltransferase appears to be present in two different genetically determined forms, one of which is more efficient than the other, as observed by Mandel (1971) in studies with isoniazid. The active metabolite of the carcinogenic aromatic amines for the induction of bladder cancer appears to be the hydroxylamine; thus, acetylation appears to be protective. Suggestive evidence to support this finding has also been obtained in humans (Lower, 1979).

METABOLIC ACTIVATION

Aromatic amines are thought to initiate tumor formation by modifying tissue macromolecules (Clayson and Garner, 1976; Miller, 1978). These amines can be transformed to metabolites that can react with proteins and nucleic acids by an initial *N*-oxidation, followed by a second activation step. The reactivity of the activated metabolites renders them unstable and limits the distance through which they are likely to exert their carcinogenic properties. Conversely, it is likely that all or only the final metabolic activation step takes place in the tissue in which tumors are induced. Consequently, the capacity of tissues to carry out these metabolic events is an important determinant of the susceptability of that tissue to a carcinogen. Furthermore, at a higher level of resolution, it is probable that the intracellular location of the metabolic activation event can be equally crucial.

The induction of liver tumors in rats by primary aromatic amines has been associated with their conversion to reactive, toxic sulfate conjugates of their hydroxamic acid derivatives (Irving, 1977). This pathway is apparently restricted to rat liver and is ineffective in the livers of female rats of some strains. The hepatocarcinogenicity of the secondary amine *N*-methyl-4-aminoazobenzene in rats appears to result from an initial *N*-oxidation and a secondary conjugation with sulfate to yield a reactive metabolite (Kadlubar *et al*., 1976).

An alternative mechanism for the metabolic activation of arylhydroxamic acids is by the formation of reactive N-acetoxyarylamines as a consequence of N,O-acyltransferase (King and Allaben, 1978). Cytosolic enzymes capable of activating N-hydroxy-AAF have been demonstrated in a wide variety of tissues from a number of species that are susceptible to the carcinogenic effects of aromatic amines. Previous studies have shown that the lactating mammary glands of the rat, like rat liver, possess an N,O-acyltransferase and that RNA adducts formed in this tissue are compatible with an acyltransferase-mediated mechanism of activation (King et al., 1979). This metabolic pathway has been implicated in tumor production in two ways. Malejka-Giganti and Gutmahn (1975) demonstrated that the direct application of N-hydroxy-AAF was more tumorigenic than either N-hydroxy-2-aminofluorene or N-2-AAF. Allaben et al. (1978) reported that direct application of the N-formyl, N-acetyl, or N-propionyl derivatives of N-hydroxy-2-aminoflurorene resulted in tumor induction that was greater than that of the N-acetyl derivative, which was also the most effective substrate with partially purified rat liver arylhydroxamic acid N,O-acyltransferase.

A third type of esterification of N-oxidized derivatives has been reported in the activation of 4-hydroxylaminoquinoline-N-oxide in incubations containing ATP-serine and seryl-adenosine-monophosphate synthetase (Tada and Tada, 1976). The activity of this system does not appear to be of general importance in the activation of other aromatic amine derivatives.

Another mechanism for activating aromatic amines is the generation of reactive metabolites via pathways involving peroxidation. Bartsch *et al.*, (1972) described the oxidation of *N*-hydroxy-*N*-2-AAF by peroxidase and hydrogen peroxide to yield the reactive ester, N-acetoxy-N-2-AAF. Subsequent studies have described the generation of radicals associated with this reaction by preparations from rat mammary gland (Floyd *et al.*, 1978).

Although adduct formation with lipids has been demonstrated in this system, reactions involving nucleic acids have apparently not been considered. A more recent variation in this area has been the generation of reactive benzidine derivatives by an arachidonic-acid-dependent, prostaglandin synthetase system obtained from rat kidney (T. Zenser, St. Louis University, personal communication). One unique feature of these findings is that the prostaglandin synthetase activation uses the free primary amines; prior *N*-oxidation is not required. The relationship of this metabolic activation pathway to the carcinogenicity of benzidine, however, remains to be explored.

Metabolism

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Chapter 3

STRUCTURE-ACTIVITY RELATIONSHIPS AMONG THE CARCINOGENIC AROMATIC AMINES

In certain instances, aromatic amines are converted in the host organism to arylhydroxamic acids or arylhydroxylamine derivatives, which are believed to be the ultimate carcinogenic forms of those amines which are carcinogens (Miller and Miller, 1976). These substances induce tumors, usually in tissues distant from their sites of administration (Clayson and Garner, 1976). The tumor site varies with the chemical, species, and strain of test animal used.

So far, there is little understanding as to why one aromatic amine attacks one tissue while another amine affects a different one. Irving (1979) discussed species and tissue differences in aromatic amine metabolism as one factor in determining the distribution of induced tumors. Other factors, such as differing tissue and species levels in prereplicative DNA repair, cell proliferation, and hormonal responsiveness, also need to be considered before a comprehensive picture of tumor distribution can be formulated. Besides the structure-activity relationships of the aromatic amines themselves, aromatic aroyl- and acylamides, aromatic hydroxylamines and hydroxamic acids, and aromatic azo, hydrazo, nitroso, and nitro compounds are also discussed in this chapter. In this discussion, aromatic amines and their derivatives are regarded as carcinogens if they significantly induce cancer in any tissue in any species.

Clayson (1953) suggested that, to be carcinogenic, an aromatic amine had to:

- possess two or three conjugated aromatic ring systems (e.g., biphenyl or naphthalene) and
- have the amino group substituted in the aromatic ring in the para position to a conjugated aromatic system.

This suggestion has been refined more recently by the clear demonstration that single-ring aromatic amines such as o-toludine may induce cancer (Weisburger *et al*., 1978), although structures of the type suggested by Clayson (1953) represent some of the more potent carcinogens.

Another important discovery has been the demonstration of the considerable carcinogenic potency of certain amino and nitroheterocyclic aromatics, such as the derivatives of 5-nitrofuran, nitrothiazole, and nitroimidazole. These compounds also have considerable importance because of their use in medicines for animals and humans, to treat parasitic infections. Because these substances are structurally dissimilar to the simpler aromatic amines, they are discussed separately.

The information on which the conclusions in this chapter are based has been reviewed by Miller and Miller (1976), Clayson and Garner (1976), and Weisburger *et al*. (1978).

SUBSTITUENTS ON THE NITROGEN

Alkylation

Alkylation of the aromatic amino group has been intensively studied only with derivatives of p-dimethylaminoazobenzene. In this particular series, the presence of at least one methyl group on the amino group is held to be essential to hepatocarcinogenicity in rats; longer alkyl groups depress activity. Detailed examination of the evidence supporting this statement reveals that few nonalkylated derivatives (analogs of p-aminoazobenzene) have been studied and that the evidence for the essential nature of the methyl group is confined mainly to 4-dimethylaminoazobenzene itself. In specific cases, such as 3-methoxy-4-aminoazobenzene or 4-(o-tolylazo)-o-toluidine, the N-methyl group is not essential, and tumors are induced. Painting p-aminoazobenzene or a range of similar chemicals on rat skin leads to skin tumors (Fare and Orr, 1965; Fare, 1966).

There is very limited evidence for the importance of an alkyl group on the nitrogen to the carcinogenic potency of aromatic amines. A methyl group may be essential to the activity of 4-dimethylaminoazobenzene and certain of its analogs in rat hepatocarcinogenesis. Dimethyl derivatives are metabolically monodemethylated to monomethylaminoazobenzene derivatives and then to the unsubstituted amines. Contrary to earlier evidence, the reverse process, arylamine methylation, is not a major metabolic pathway (Scribner et al., 1965). Higher alkyl groups generally result in chemicals with lower carcinogenicity than do those provided by the free amines. Monoalkylaromatic amines can be converted to the corresponding nitrosamines in the presence of nitrous acid at an acid pH.

Arylation

Arylation of the amino group to diarylamines or triarylamines is believed to abolish carcinogenic activity, although the evidence for this conclusion is tenuous. Phenyl-2-naphthylamine has been studied intensively because of its use as a substitute for 2-naphthylamine, a rubber-compounding ingredient. The urine of humans and dogs exposed to this apparently noncarcinogenic chemical contains low levels of free 2-naphthylamine (Batten and Hathway, 1977; Kummer and Tordoir, 1975;). At this time, there is no explanation for this finding--whether the 2-naphthylamine is liberated within the host or is an artifact of urine collection, or whether there is a similar degradation of other diarylamines. The biologic significance of this observation can be judged from the fact that the amount of urinary 2-naphthylamine found after exposing humans to 10 mg of phenyl-2-naphthylamine is equivalent to that in the smoke of 4-40 cigarettes. Hoffmann et al. (1969) found 1 µg of 2-naphthylamine in the smoke of 40 cigarettes.

Acylation

Acetylation and deacetylation of aromatic amines are common metabolic reactions in most species, except dogs, which lack the ability to acetylate these chemicals. Consequently, aromatic amines and acetamides generally possess similar carcinogenic potencies. Higher homologs of the acetyl group do not appear to have been investigated. _N_-2-fluorenylformamide is less potent than the acetyl derivative (Miller _et_ _al_., 1962).

Aroyl Derivatives

Aroyl derivatives have been examined, particularly in the 2-fluorenylamine series. Although _N_-2-fluorenylbenzamide is inactive, the corresponding _N_-hydroxy-2-fluorenylbenzamide is carcinogenic. This finding suggests that aroylation blocks _N_-hydroxylation, the essential activation route for this carcinogen. The benzenesulfonamide derivatives of 2-fluorenylamine are likewise inactive, but its _N_-hydroxy derivative is carcinogenic (Gutmann _et_ _al_., 1967).

N-Hydroxylation

_N_-Hydroxylation of aromatic amines or of their amides is generally regarded as the first step toward their metabolic activation. If active, aromatic hydroxylamines or hydroxamic acids are more potently carcinogenic than are the non-_N_-hydroxylated chemicals; however, the demonstration of this increase in potency may require careful selection of a system (Miller _et_ _al_., 1964). In specific instances, the _N_-hydroxylated compound demonstrates activity, and the parent compound does not. 1-Naphthylamine, for example, is not demonstrably carcinogenic if free from the potently carcinogenic 2-isomer; however, the corresponding N-hydroxy derivative, is carcinogenic (Radomski et al., 1971).

Esters of N-Hydroxy Derivatives

Esters of N-Hydroxy derivatives of aromatic amines have been regarded as the ultimate carcinogenic forms of the aromatic amines. Some of these derivatives are highly genotoxic, if they can be delivered to the test system and the critical receptors before they interact with other possible targets such as thiols. The less polar model esters (such as N-acetoxy-2-fluorenylacetamide) are thus more readily shown to be carcinogenic than are the more polar derivatives (such as the sulfate ester of N-hydroxy-2-fluorenylacetamide).

Different esters appear to vary in their ability to act as leaving groups in the production of the arylnitrenium ion. For example, the O-glucuronic acid ester of N-hydroxy-2-fluorenylacetamide does not appear as capable of producing the nitrenium ion as do either the acetoxy or sulfate esters (Irving and Wiseman, 1971).

Azo Compounds and Hydrazo Compounds

These compounds are reduced in the anaerobic portions of the gastrointestinal tract, or by the tissue enzyme, azoreductase, to compounds, each of which contains an amino group. Several examples of azo compounds, themselves carcinogenic and capable of reduction to carcinogenic aromatic amines have been reported. Bonser *et al*. 1954 and Weisburger *et al*. (1978) reported that 1-(*o*-tolyl)azo-2-naphthol is carcinogenic and may be reduced to *o*-toluidine, which is also carcinogenic. The high carcinogenic potency of a series of dyes (Direct Black 38, Direct Brown 95, and Direct Blue 6), which are capable of reduction to benzidine, has been reported by the National Cancer Institute (1978).

Aromatic C-Nitroso Compounds

These compounds are of interest because of their easy conversion to arylhydroxylamines. Certain *C*-nitroso compounds are effective nitrosating agents.

THE RING SYSTEM AND THE POSITION OF THE AROMATIC AMINO GROUP

Ring-substituted amino derivatives of most substances containing one, two, three, and possibly four aromatic rings may be carcinogenic. The placement of the amino group is the critical factor. Thus, 2-naphthylamine, 2-acetamidofluorene, 2-anthramine, and 2-phenanthrylacetamide are potent carcinogens; 1-naphthylamine, 3-acetamidofluorene, 9-anthramine, and 9-phenanthrylacetamide are not. The presence of a large conjugated group para to the amino group appears to enhance carcinogenic activity but is not a requirement for this property, as is clearly demonstrated by the fact that single-ring aromatic amines and derivatives of methylenedianiline are carcinogenic (International Agency for Research on Cancer, 1974a,b,c; Weisburger *et al*., 1978).

The basic ring system for carcinogenic aromatic amines may be entirely carbocyclic (2-naphthylamine, 4-biphenylamine), may show limited numbers of heteroatoms (3-aminodiphenylene oxide, 4-N-quinoline-1-oxide), or may be highly heterocyclic (nitridazole, metronidazole). The heterocyclic chemicals are discussed separately later in this chapter.

Ring substituents on the carcinogenic potential of aromatic amines are subdivided into

Analogs of p-dimethylaminoazobenzene
Aromatic amines with single amino groups
Analogs of the phenylenediamines
Analogs of benzidine.

This subdivision is necessary because the various types of carcinogenic aromatic amines appear to be affected differently by substituents.

Analogs of p-Dimethylaminoazobenzene

Analogs of p-dimethylaminoazobenzene have been extensively studied for their induction of rat liver tumors. There are five different positions available for monosubstitution. The effects of the substituents on carcinogenic activity are presented in Tables 3-1, and 3-2.

Table 3-1

Effect of Substituents on the Carcinogenic Activity of N,N-Dimethyl-p-phenylzaoaniline[a]

Substituent	2	3	2'	3'	4'	2,3'	2,4'	2,6'	3',4'	3',5'	2',4',
$-CH_3$	-	+	+	+	+	-	-		+	-	
$-C_2H_5$			-	+	+				+		
$-CF_3$			-	-	-						
-F	+	+	+	+	+			-		+	+
-Cl			+	+	+				+		
-Br				-							
-OH	-	-	-	-	-						
$-OCH_3$	±	+[b]	+	+	+						
$-OC_2H_5$				±							
$-NO_2$			+	+	-						
$-NH_2$					-						
$-SO_3H$					-						
$-CO_2H$			±[c]	±	-						

[a] From Clayson and Garner, 1976, with permission.

[b] Ear duct, skin, and intestinal tumors, but no hepatomas.

[c] Bladder papillomas; hepatomas possibly induced.

Table 3-2

Liver Carcinogenicity of N, N-Dimethyl-p-phenylazoanilines in Rats [a]

Positive

- *N*,*N*-Dimethyl-4(4′-benzimidazolylazo)aniline
- *N*,*N*-Dimethyl-4(6′-benzthiazolylazo)aniline
- *N*,*N*-Dimethyl-4(7′-benzthiazolylazo)aniline
- *N*,*N*-Dimethyl-4[4′(2′,6′-dimethylpyridyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4(6′-*IH*-indazylazo)aniline
- *N*,*N*-Dimethyl-4(4′-isoquinolinylazo)aniline
- *N*,*N*-Dimethyl-4(5′-isoquinolinylazo)aniline
- *N*,*N*-Dimethyl-4(7′-isoquinolinylazo)aniline
- *N*,*N*-Dimethyl-4(5′-isoquinolyl-2′-oxide)azoaniline
- *N*,*N*-Dimethyl-4[4′-(2′,5′-lutidyl)azo]aniline
- *N*,*N*-Dimethyl-4[4′-(2′,6′-lutidyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[3′,5′-lutidyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[4′(2′-methylpyridyl)azo]aniline
- *N*,*N*-Dimethyl-4[2′-methylpyridyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[4′-(3′-methylpyridyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[4′(3′-methylpyridyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[4′(2′-methylpyridyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[4′(2′-methylpyridyl-1′-oxide)azo]*o*-toluidine
- *N*,*N*-Dimethyl-4[5′(3′-methylquinolyl)azo]aniline
- *N*,*N*-Dimethyl-4[5′(6′-methylquinolyl)azo]aniline
- *N*,*N*-Dimethyl-4[5′(7′-methylquinolyl)azo]aniline
- *N*,*N*-Dimethyl-4[5′(8′-methylquinolyl)azo]aniline
- *N*,*N*-Dimethyl-4(2′-naphthylazo)aniline
- *N*,*N*-Dimethyl-4[3′-picolyl-1′-oxide)azo]-*o*-toluidine
- *N*,*N*-Dimethyl-4[3′-picolyl-1′-oxide)azo]-*m*-toluidine
- *N*,*N*-Dimethyl-4[4′-pyridyl-1′-oxide)azo]-2,3-xylidine
- *N*,*N*-Dimethyl-4[(4′-pyridyl-1′-oxide)azo]-2,5-xylidine
- *N*,*N*-Dimethyl-4[(4′-pyridyl-1′-oxide)azo]-3,5-xylidine
- *N*,*N*-Dimethyl-4(3′-pyridylazo)aniline
- *N*,*N*-Dimethyl-4[4′-pyridyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4(5′-quinaldylazo)aniline
- *N*,*N*-Dimethyl-4(3′-quinolylazo)aniline
- *N*,*N*-Dimethyl-4(4′-quinolylazo)aniline
- *N*,*N*-Dimethyl-4(5′-quinolylazo)aniline
- *N*,*N*-Dimethyl-4(6′-quinolylazo)aniline
- *N*,*N*-Dimethyl-4[(4′-quinolyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[(5′-quinolyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[(6′-quinolyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4(5′-quinolylazo)-*m*-toluidine
- *N*,*N*-Dimethyl-4(2′-quinoxalylazo)aniline
- *N*,*N*-Dimethyl-4(5′-quinoxalylazo)aniline
- *N*,*N*-Dimethyl-4(6′-quinoxalylazo)aniline

Negative

- 2-Dimethylamino-5(phenylazo)pyridine
- *N*,*N*-Dimethyl-4(5′-benzimidazolylazo)aniline
- *N*,*N*-Dimethyl-4(2′-dibenzofuranylazo)aniline
- *N*,*N*-Dimethyl-4(1′-dibenzothienylazo)aniline
- *N*,*N*-Dimethyl-4(2′-dibenzothienylazo)aniline
- *N*,*N*-Dimethyl-4(3′-dibenzothienylazo)aniline
- *N*,*N*-Dimethyl-4(3-dibenzothienylazo)aniline
- *N*,*N*-Dimethyl-4(4′-benzthiazylazo)aniline
- *N*,*N*-Dimethyl-4(5′-benzthiazylazo)aniline
- *N*,*N*-Dimethyl-4(2-fluorenylazo)aniline
- *N*,*N*-Dimethyl-4(3′-*IH*-indazylazo)aniline
- *N*,*N*-Dimethyl-4(4′-*IH*-indazylazo)aniline
- *N*,*N*-Dimethyl-4(5′-*IH*-indazylazo)aniline
- *N*,*N*-Dimethyl-4(7′-*IH*-indazylazo)aniline
- *N*,*N*-Dimethyl-4[2′-(4′-methylpyridyl)azo]aniline
- *N*,*N*-Dimethyl-4[2′-(6′-methylpyridyl)azo]aniline
- *N*,*N*-Dimethyl-4-(7′-quinolylazo)aniline
- *N*,*N*-Dimethyl-4-(8′-quinolylazo)aniline
- *N*,*N*-Dimethyl-4[2′-quinolyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[3′-quinolyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[7′-quinolyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[8′-quinolyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[2′-(4′-methylpyridyl-1′-oxide)azo]aniline
- *N*,*N*-Dimethyl-4[2′-(6′-methylpyridyl-1′-oxide)azo]aniline

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[a] From Clayson and Garner, 1976, with permission.

Aromatic Amines Containing Single Amino Groups

These aromatic amines have a number of similarities that lead to structure-activity relationships.

Methyl Substitution. Especially when ortho to the amino group, methyl substitution appears to enhance activity, as is demonstrated by comparing the carcinogenic potential of 2',3-dimethyl-4-aminobiphenyl to 4-aminobiphenyl, or of 3-methyl-2-amino-naphthylamine to 2-naphthylamine. The effect of methyl groups in other positions in the 4-aminobiphenyl system was extensively studied by Walpole and Williams (1958). Methyl groups in both ortho positions, as with 2,6-dimethylaniline (2,6-xylidine), may be deactivating (National Cancer Institute, in press).

Methoxyl Substitution. Ortho substitution of a methoxyl group has a varied effect. Thus, 3-methoxy-4-aminobiphenyl and 1-methoxy-2-fluorenylacetamide are just as, or even more, potent than the parent carcinogen in rats, although 3-methoxy-2-fluorenylacetamide was found to be without activity. Similar results were reported for the free amines. Other methoxyl substitutions also make the molecule more carcinogenic as, for example, in the case of *p*-cresidine as compared to aniline or 7-methoxy-2-fluorenylacetamide as compared to 2-fluorenylacetamide.

Halogen Substitution. This is best illustrated by using fluorine substitution to block metabolic detoxification of aromatic amines. Fluorine enhances carcinogenicity, as illustrated by the potent carcinogens 4'-fluoro-4-biphenylamine and 7-fluoro-2-fluorenylacetamide. Most fluoro derivatives of N-2-fluorenylacetamide have been tested (Miller *et al.*, 1962). Insufficient data are available to permit a useful statement on other halogen-substituted aromatic amines.

Polar Group Substitution. It is generally held that substitution with sulfonic acid, carboxylic acid, or phenolic groups diminishes the carcinogenicity of aromatic amines. There are, however, few published animal studies to support this viewpoint. Epidemiology can do little to provide useful information because the noxious parent aromatic amines are usually used in proximity to the polar derivatives.

Anologs of Phenylenediamines

More than 14 phenylenediamines or the corresponding nitro compounds have been adequately tested for carcinogenicity (National Cancer Institute, 1978a,b,c,d,e,f; 1979a,b; Weisburger *et al.*, 1978). A limited number of these agents are effective carcinogens, including 2,4-diaminotoluene, 5-nitro-*o*-anisidine, and 4-chloro-*o*-phenylenediamine; others, under the test conditions used, exhibited more marginal carcinogenicity, including 2-*N*-*p*-phenylenediamine, 2,5-diaminotoluene sulfate, 2,4-dinitrotoluene, and tetrafluorometaphenylenediamine. The remainder were inactive.

This evidence demonstrates that derivatives of each of the three positional isomers of phenylenediamine or the corresponding nitro compounds may exhibit carcinogenicity. So far as the limited data permit a decision, it appears that the carcinogenicity of phenylenediamine, is activated by the presence of a methyl or methoxyl group ortho to one of the amino groups in the same way as are the aromatic monoamines. Blocking metabolically important positions for detoxification, as in tetrafluorometaphenylenediamine, may also be important.

Analogs of Benzidine

Information obtained mainly from studies of rats indicates that benzidine is more carcinogenic than o-tolidine, o-dianisidine, or o-dichlorobenzidine. In a limited study, 3,5,3',5'-tetramethylbenzidine appears inactive. Overall, benzidine derivatives appear to behave differently in their structure-activity relationships than do either the aromatic monoamines or derivatives of methylene(bis-aniline), in which o-methyl- or o-chloro-substitution appears to enhance carcinogenicity (Munn, 1967).

NITRO- AND AMINO-AROMATIC HETEROCYCLIC COMPOUNDS

In the furan, thiophene, imidazole, and thiazole series, both of the unsaturated bonds provide a pair of π electrons, and one of the hetero atoms provides a lone pair of electrons to form the aromatic sextet. Thus, the amino and nitro derivatives of these heterocyclic resemble the carbocyclic analogs in various ways, including cancer induction. The initial discovery by Price et al. (1966) and Stein et al. (1966) has been followed by the bioassay of many environmentally important substances of this type (Table 3-3). The nitrofuran derivatives have been fully reviewed by Bryan (1978).

It is apparent that many of the nitroheterocyclic compounds are potent carcinogens with demonstrable effects in many tissues. The amino-substituted analogs have been much less intensively studied. Structure-activity relationships are difficult to evaluate because of the competing effects of the hetero atoms, the substitutents, and the various conjugated aromatic systems. It does, however, appear that compounds with two conjugated aromatic ring systems are, when carcinogenic, more potent than are single-ring systems. Thus, the low activity of the single-ring substance, metronidazole (Flagyl®), can be compared to the considerable carcinogenic potency of niridazole, which possesses two ring systems (Bulay et al., 1977; Rustia and Shubik, 1972).

CONCLUSIONS

Many aromatic amino and nitro compounds can induce cancer in humans or animals. Unless polar groups, such as sulfonic acid or carboxylic acid substituents, are present in the molecule, it is possible that these chemicals are potentially carcinogenic. Fortunately, however, the most potent aromatic amine carcinogens

Table 3-3
Carcinogenicity of 2-Nitrofuryl Compounds and Related Substances [a]

Compound	Species	Route[a]	Adequacy[b]	Tumors Induced in[c]: Local	Bladder	Kidney	Liver	Intestine	Ear Duct	Breast	Other
2-(2,2-Dimethylhydrazino)-4-(5-nitro-2-furyl)thiazole	rat	O	A	–	–	–	–	–	–	+	salivary gland, various other tissues
	mouse	O	B	–	?	?	–	–	–	–	stomach
Formic acid, 2-[4-(5-nitro-2-furyl)-2-thiazolyl]hydrazide	rat	O	B	–	–	+	?	–	–	+	lung? skin? stomach
	hamster	O	B	–	+	–	–	–	–	–	stomach?
	mouse	O	B	–	–	–	–	–	–	+	lung?
2-Hydrazino-4-(5-nitro-2-furyl)thiazole	rat	O	B	–	–	?	–	–	–	+	stomach?
	mouse	O	B	–	–	–	–	–	–	+	stomach?
2-Hydrazino-4-(4-nitrophenyl)thiazole	rat	O	B	–	–	–	–	–	–	+	skin? salivary gland?
	mouse	O	B	–	–	–	–	–	–	–	stomach?
N-[4-(5-Nitro-2-furyl)-2-thiazolyl] formamide	dog	O	B	–	+	–	–	–	–	+	gall bladder
	rat	O	A	–	+	+	–	–	–	+	–
	hamster		B	–	+	–	–	–	–	–	stomach
	mouse	O	B	–	+	–	–	–	–	–	lung? leukemia?
5-Acetamido-3-(5-nitro-2-furyl)-6*H*-1,2,4-oxadiazine	rat	O	B	–	–	–	+	–	–	–	lung, mesentery (hemangio-endothelio-sarcomas)
5-Nitro-2-furaldehyde semicarbazone	rat	O	B	–	–	–	–	–	–	?	–
N-[4-(5-Nitro-2-furyl)-2-thiazolyl] acetamide	rat		B	–	–	+	–	–	–	+	lung, salivary gland
	hamster		B	–	+	–	–	–	–	–	–
	mouse		B	–	–	–	–	–	–	–	leukemia, stomach
1-(5-Nitro-2-furylidene)amino hydantoin	rat	O		–	–	–	–	–	–	–	–
4-Methyl-1-[5-nitrofurylido)amino]-2-imidazolidinone	rat	O	B	–	–	–	–	–	–	+	–
1-5-Morpholinomethyl-3-[(5-nitro-2-furylidene)amino]-2-oxazolidine	rat	O	B	–	–	–	–	–	–	+	lymphoma
1-(2-Hydroxyethyl)-3-[5-nitrofurylidene) amino]-2-imidazolidine	rat	O	B	–	–	–	–	–	–	+	–
1-[(5-Nitrofurylidene)amino]-2-imidazolidinone	rat	O	B	–	–	–	–	–	–	+	lymphoma
5-Nitro-2-furamidoxime	rat	O	B	–	–	–	–	–	–	–	–
4,6-Diamino-2-(5-nitro-2-furyl)-s-triazine	rat	O	B	–	–	–	–	–	–	+	–
N,N'-[6-(5-Nitro-2-furyl)-s-triazine-2,4-diyl]-bisacetamide	rat	O	B	–	–	–	–	–	–	+	–
Hexamethylmelamine	rat	O	B	–	–	?	–	–	–	?	–
2-Hydrazino-4-phenylthiazole	rat	O	B	–	–	–	–	–	–	–	–
D-(−)-Threo-1-(*p*-nitrophenyl)-2-dichloroacetamido-1,3-propanediol	rat	O	B	–	–	–	–	–	–	–	–
1-(2-Hydroxyethyl)-2-methyl-5-nitroimidazole	rat	O	B	–	–	–	–	–	–	+	–
1,2-Dimethyl-5-nitroimidazole	rat	O	B	–	–	–	–	–	–	+	–
2-Amino-5-phenyl-2-oxazolin-4-one + $Mg(OH)_2$	rat	O	B	–	–	–	–	–	–	?	–
3-Aminotriazole	rat	O	A	–	–	–	+	–	–	–	thyroid
	mouse	O	A	–	–	–	+	–	–	–	thyroid
1-(2-Hydroxy)ethyl-2-methyl-5-nitroimidazole	mouse	O	B	–	–	–	–	–	–	–	lymphoma, lung

[a] O, oral.
[b] A, tested in more than one institute; B, evidence less than convincing.

[c] +, tumors reported; –, tumors not reported; ?, evidence equivocal.

[a] From Clayson and Garner, 1976, with permission.

appear to possess certain specific structural characteristics, such as

- one, two, or three conjugated aromatic ring systems,
- an aromatic amino group substituted in the position para to the conjugated aromatic system, or
- groups such as methyl, methoxyl, or fluorine substituted in specific positions relative to the amino group.

Aniline, as the simplest aromatic amine, might be considered the reference chemical for structure-carcinogenic activity relationships in this series. However, although it has induced cancer in rats (National Cancer Institute, 1978g), but not in mice, it has shown negative results in mutagenicity tests. If norharmon is present in Salmonella tests the results are positive (Nagao et al., 1977). Thus, it may induce splenic hemangiosarcoma and other sarcomas by a mechanism different from that by which other aromatic amines induce their effects. For example, the methemoglobinemia induced by aniline may stress the spleen, which removes debris in red blood cells from the circulation, thereby setting up the conditions for splenic tumorigenesis. p-Chloroaniline induces similar cancers and, likewise, induces high levels of methemoglobinemia (National Cancer Institute, 1979d).

p-Cresidine (2-methoxy-5-methylaniline) induced bladder carcinomas and olfactory neuroblastomas in rats and bladder carcinomas and hepatocellular carcinomas in mice (National Cancer Institute, 1979c). p-Cresidine produced positive results in microbial mutagenicity tests. There is no reason to doubt that genotoxic factors play a role in inducing these tumors and, as they occur at sites usual for aromatic amines, p-cresidine should be regarded as a potent carcinogen. The ortho methoxyl group is probably responsible for enhancing the activity of this amine.

2,4-Diaminotoluene (National Cancer Institute, 1978h) induced hepatocellular carcinomas in male and female rats and in female mice. In female rats, mammary adenocarcinomas were induced. The substance is mutagenic in microbial systems. It is a genotoxic carcinogen, and its activity is enhanced by the methyl group ortho to the amino group.

Methylene-bis(o-chloraniline) is clearly more carcinogenic than is methylenedianiline (Munn, 1967). It provides an example of the enhancing effects of ortho chloro substitution; ortho methyl substitution also enhances carcinogenicity.

Furazolidine is a borderline carcinogen. This is to be expected from its single-ring structure, as activity in the nitro heterocyclic series is highest when two aromatic heterocyclic ring systems exist in the molecule.

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Chapter 4

CARCINOGENIC POTENCY AND RISK ESTIMATION

In this chapter the committee uses information concerning the carcinogenicity of aromatic amines to suggest a means for expressing concepts of carcinogenic potency and for estimating risk.

Aromatic amines are excellent candidates for risk assessment. They have been assayed for carcinogenicity in several animal species and strains, and some members of this class (2-naphthylamine, benzidine, 4-aminobiphenyl, and probably phenacetin) are indeed bladder tumor inducers in humans. 1-Naphthylamine, as manufactured at one time, was associated with bladder cancer in workers; this was probably due to a high level (4-10%) of contamination with 2-naphthylamine. Of course, there are no reliable data concerning the level of adventitious human exposure in the workplace or elsewhere, although the amount of chemical administered to laboratory animals is readily determined. Limited data may possibly be obtained from studying iatrogenic carcinogens, e.g., 2-naphthylamine mustard (Thiede and Christensen, 1975), but in such cases, animal data are usually deficient.

CARCINOGENIC POTENCY

The potency of a carcinogen depends on three factors: the dose of carcinogen required to induce tumors, the time to tumor induction, and the percentage of tumor response. For purposes of the following discussion the committee has defined potency as

$$\text{potency} = 7 - \log d_{E50}$$

where d_{E50} is the dose expressed in µmol/kg body weight/week required to induce tumors in 50% of the animals in a lifetime experiment. This dose is expressed as a logarithm in order to compress the range of values to a readily understandable format and to avoid undue emphasis on small differences in potency that lack biologic significance. The negative of the logarithm is used because potency is inversely related to the dose required to induce tumors, and the number 7 is used to bring all values to a readily comprehensible positive form.

As calculated according to the above criteria, typical potencies of a number of carcinogens that induce liver or bladder tumors in rat or mouse are shown in Table 4-1. The data illustrate the range of potency from the most potent (aflatoxin B1) to the least potent (trichloroethylene and saccharin) carcinogens. The calculations used to determine these values are admittedly approximate. For example, an approximation has been made that in attaining a 50% dose response, the dose experimentally shown to give a 25% tumor incidence has been doubled; likewise, doses that lead to tumors in half the lifespan of the animal have been halved to calculate d_{E50}. These assumptions may well be adequate because the logarithmic scale compresses small differences. The values for the lifespan of several test species and their food and water consumption requirements are compared in Table 4-2. These are the values used for d_{E50} calculations. Better approximations would lead to more accurate potency values.

Table 4-1

The Potency of a Range of Carcinogens to Rat or Mouse Liver Following Continuous Feeding

Chemical	Species	$\log_{10} d_{E50}$ (mg/kg/week)	Potency
Aflatoxin B1	Rat	0.67	9.18
Michler's ketone	Rat	4.88	4.62
Dimethylnitrosamine	Rat	4.90	4.00
Carbon tetrachloride	Rat	5.27	3.87
2-Aminoanthraquine	Rat	6.72	4.44
Trichloroethylene	Mouse	7.03	2.12

Table 4-2

Factors Used for Calculating d_{E50} in Animals

Factors(s)	Mouse	Rat	Dog
Lifespan (years)	2.5	3.0	10
Food (g/day)	4-6	12-15	300-500
Drinking water (ml/day)	2.1	20.0	NK[a]
Gestation (days)	21.0	21.0	63
Weight (g)	25-40	100-500	10,000

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[a] NK = not known

Certain features of this potency derivation need emphasis. First, the dose rate (μmol/kg body weight/week) is used in preference to the total dose to avoid giving the impression that long-lived species are less sensitive to carcinogens than are shorter-lived species. For example, it has been accepted that dogs are sensitive to the carcinogenicity of 2-naphthylamine, and that mice are less sensitive. This finding is shown in Table 4-3. Total exposures (450-500 weeks in dogs compared to 100 weeks in mice) indicates that dogs and mice are more similarly sensitive to this chemical.

The potency as calculated here defines one point on the tumor incidence-dose curve. If one accepts the linear one-hit model (National Academy of Sciences, 1976) of carcinogenesis, which, is probably not universally accurate, as shown later in this chapter, the potency value effectively defines the slope of the dose-response curve. Whatever tumor incidence-dose model is used, the potency values discussed here have a considerable advantage in that only they may be derived without excessive data extrapolation. In many cases, where two or more doses of a carcinogen have been used, interpolation rather than extrapolation may be required.

Tables 4-4 and 4-5 show the potency values for 4-aminobiphenyl and methylene-bis(o-chloraniline) in different species. Although the potency of a carcinogenic aromatic amine in different species appears to lie within two orders of magnitude, this impression, to a certain extent, is false; species that fail to respond to an aromatic

Table 4-3

Potency of 2-Naphthylamine in Different Species Following Oral Administration

Species	$\log_{10} d_{E50}$ (mg/kg/week)	Potency
Dog	4.92	4.34
Mouse	5.23	3.93
Rat	6.72	2.74
Hamster	6.72	2.14

Table 4-4

Potency of 4-Aminobiphenyl Following Administration to Different Species

Species	Route of Administration	Tissue	Potency
Dog	Oral	Bladder	6.22
Mouse	Gavage	Liver	4.52
Rat	Subcutaneous	Intestine	4.37

Table 4-5

Potency of Methylene-bis(o-chloraniline)in Various Species Following Oral Administration

Species	Tissues	$\log_{10} d_{E50}$ [a] (mg/kg/week)	Potency
Dog	Bladder	4.57	4.86
Rat[b] (adequate diet)	Lung	4.81	4.57
	Breast	5.15	4.25
	Zymbal's gland	4.85	4.58
	Liver	4.72	4.70
Rat[b] (low protein diet)	Lung	5.08	4.35
	Breast	5.48	3.95
	Zymbal's gland	5.18	4.25

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[a] Each value of d_{E50} is calculated as life correction x tumor yield correction x dose x 105 µg/kg/week. It is assumed that rats live 95-104 weeks and consume an average 105 g/food/week and that dogs live an average of 9 years, or 468 weeks.

[b] Only male animals were used.

amine are omitted from the tables. It is not possible to decide whether these nonresponsive species are completely insensitive to the particular agent or are sensitive at a level too low to be detected in the animal bioassay. The cutoff point for the sensitivity of a carcinogenesis bioassay is determined first by the toxicity of the chemical being tested and, second, by the maximum amount of test substance it is reasonable to give to an animal during any one period. The use of the maximum-tolerated dose, as recommended in the bioassay protocols of the National Cancer Institute/National Toxicology Program (Sontag *et al*., 1976), ensures that the highest feasible dose level is used, but that this level may lead to abnormal results due to the intervention of the agent's toxic properties in the carcinogenic process. However, substances with a very low level of toxicity may, if the maximum tolerated dose is used, be administered at levels that interfere physiologically with the host, for example, by inducing nutritional imbalance, and similarly lead to difficulty in interpreting results.

At present, it is not possible to predict with confidence that the demonstration of potency of a carcinogen in one animal species means the carcinogen will be as potent in another species. Thus, it may be prudent to assume (since it cannot be tested in humans) that humans are at least as sensitive to a carcinogen as are the most sensitive species. Consequently, 2-naphthylamine in humans could be assumed to be as potent as it is in dogs (Table 4-3) and methylene-*bis*-(*o*-chloraniline) to be as potent in humans as it is in rats (Table 4-5). Table 4-6 shows the carcinogenic potency of N,2-fluorenylacetamide in different species and strains of test animals. Ways of predicting carcinogenic potency based on the present knowledge of the mechanisms of carcinogenesis urgently need to be expanded. Crouch and Wilson (1979) discuss interspecies differences in carcinogenic potency, including humans; however, they do not describe how they calculate potency to humans in the absence of reliable exposure data.

HIGH- TO LOW-DOSE EXTRAPOLATION

It is now generally regarded as prudent to assume, when extrapolating data from high carcinogenic dose rates in animals to low-dose exposure in humans, that the mathematically simple linear one-hit model is appropriate even if the limited experimental evidence does not necessarily support this conclusion. The relevance of this model, which implies that a single carcinogen-critical receptor interaction is involved, has not been seriously questioned, although it may prove unsuitable in many cases.

Chemical carcinogenesis is a multifaceted process. It is generally accepted that the procarcinogen has to be metabolically activated to the ultimate or reactive form, and the ultimate carcinogen then has to interact with its critical target. When the critical target is DNA, this interaction is followed by DNA replication to "lock in" genetic damage, or by DNA repair to restore

Table 4-6

The Carcinogenic Potency of N,2-Fluorenylacetamide in Different Species and Strains of Test Animals[a]

Species	Route	Site of Tumor	Potency
Dog	Diet	Liver, bladder	4.5
Rabbit	Gavage	Bladder, ureter	4.46
Hamster	Diet	Gall bladder	4.29
Rat			
Slonaker (M+F)[b]	Diet	Bladder	4.40
Wistar (M)	Diet	Liver	5.14
Wistar (F)	Diet	Breast	5.03
Piebald (M+F)	Diet	Intestine	4.93
Mouse			
BALB/c (F)	Diet	Liver	4.17
BALB/c (F)	Diet	Bladder	4.17

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[a] Some evaluations based on early studies.

[b] M = male; F = female.

the cells to quasi-normality, and the damaged cells or tumor progenitor cells then undergo various biologic interactions before frank clinical neoplasia is observed. It is difficult to conceive all these changes involving only a single interaction between the carcinogen and its critical receptor(s)

The simplest biologic model of carcinogenesis is the two-stage hypothesis, proposed by Berenblum and Shubik (1947a,b; 1949) in mouse skin, and now being shown applicable for many other tissues such as liver, bladder, and pancreas. This model may not effectively describe what may well be a more complex multi-stage process. Initiation and promotion are separate processes induced by different agents. Therefore, they are independent of each other. A complete carcinogen, however, is capable of both initiation and promotion. Since both processes are presumably dose dependent, an exponential rather than linear relationship should exist between dose of carcinogen and tumor response. A linear relationship may be applicable for pure initiators or pure promoters.

The validity of these suggestions is suggested by the large scale (ED_{01}) experiment conducted by the National Center for Toxicological Research (Staffa and Mehlman, 1979), in which low levels of N,2-fluorenylacetamide were fed to BALB/c mice. The liver tumor yield was apparently related linearly at low doses to the carcinogen dose; the bladder tumor yield was not (Figure 4-1).

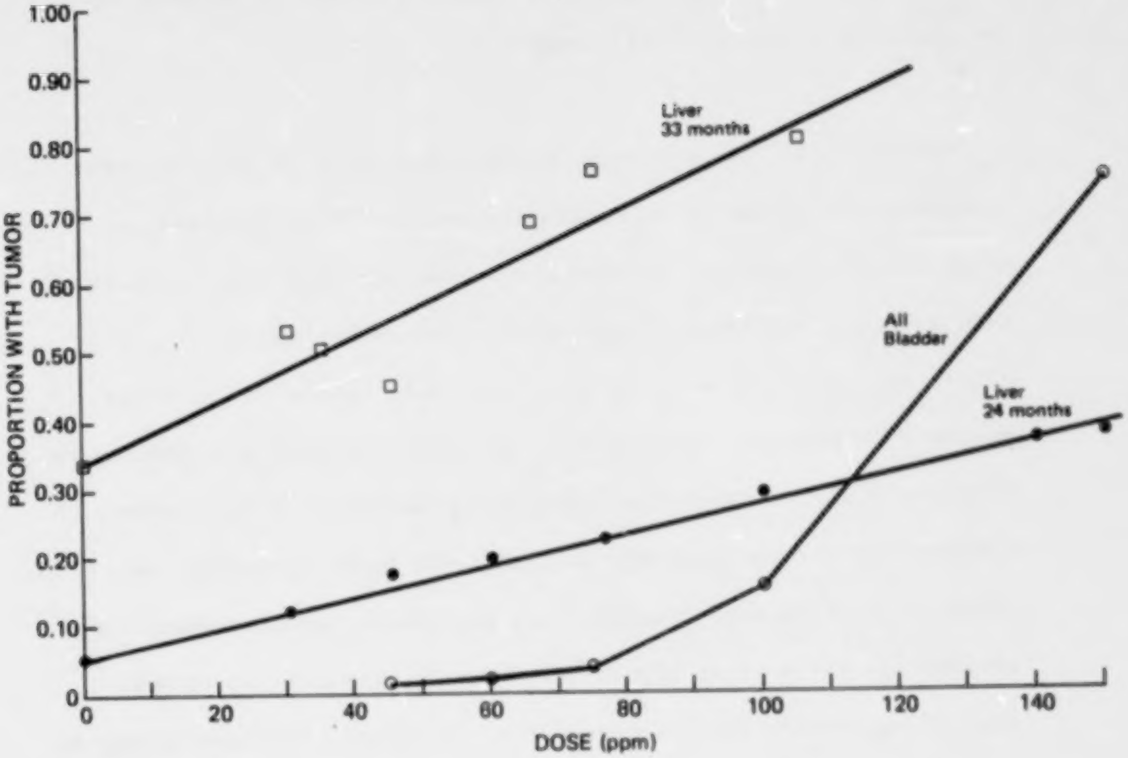

Figure 4-1 Comparison between liver and bladder tumor yield in response to different doses of N,2-fluorenylacetamide given over 33 months. These data are for BALB/c mice, (Staffa and Mehlman, 1979).

Between 24 and 33 months, BALB/c mice developed a significant incidence of naturally occurring liver tumors, which the carcinogen enhanced. The naturally occurring incidence of bladder tumors was very low. Thus, one explanation of the dose-response relationships exhibited is that the carcinogen must act as both initiator and promoter in bladder carcinogenicity to give a tumor incidence-dose curve that is very differently shaped than that for the liver, where only promotion occurs.

These observations could mean that, although a linear dose-response curve may be appropriate in specific cases (possibly with pure initiators or with pure promoters acting on an appreciable spontaneous tumor yield), it may be inappropriate in other cases. The use of linear dose-response relationships may indicate a level of risk higher than actually occurs. This result is possibly beneficial in that it provides estimates at a time when methods for risk estimations are little understood. As risk assessment techniques become more precise it may become possible to make estimates with greater accuracy.

The use of these statistical models to estimate possible risk to humans at very low exposures is filled with uncertainty. Studies with animals usually involve exposure to a high level of a single carcinogen and, sometimes, just one modifying agent. In the real, nonexperimental world, humans are exposed to a wide range of carcinogens and carcinogenesis-modifying agents, which may enhance or inhibit cancer development due to low levels of a particular agent. The suggestion that a given agent will induce, for example, one tumor in a population of a million is, under these conditions, trite speculation.

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Chapter 5

EPIDEMIOLOGIC ASPECTS OF EXPOSURE TO AROMATIC AMINES

The studies of Case *et al*.(1954a,b) in Great Britain provided the first systematic evidence of the carcinogenicity of specific aromatic amines. More recently, Clayson (1976), Clayson and Garner (1976), and the working group for the International Agency for Research on Cancer (1972, 1974a, 1978a) have concluded that bladder cancer is caused in workers who manufacture and use 2-naphthylamine, benzidine, and 4-aminobiphenyl. It is difficult to identify persons exposed only to 1-naphthylamine, auramine, magenta, and *N*-phenyl-2-naphthylamine (International Agency for Research on Cancer, 1972, 1974a, 1978a); thus, it has not been possible to determine whether any one compound alone is carcinogenic in humans. It is generally accepted that aniline has not been shown to cause bladder cancer in humans (International Agency for Research on Cancer, 1974a).

The data from the studies of Case *et al*. are presented in Table 5-1. The largest increase in fatalities occurred among men exposed to a mixture of compounds. Increased mortality rate is statistically significant and has been interpreted as causal for 2-naphthylamine and benzidine. The increased mortality rate among workers exposed to 1-naphthylamine is complicated by the fact that 1-naphthylamine contains 4-10% of 2-naphthylamine.

Table 5-1

Observed and Expected Deaths from Bladder Cancer According to Class of Exposure to Specific Substances[a],[b]

Class	Observed	Expected
Aniline without benzidine, naphthylamine, magenta, or auramine contact	1	0.83
Aniline with magenta contact	3	0.13
Aniline with auramine contact	6	0.45
Benzidine	10	0.72
1-Naphthylamine	6	0.70
2-Naphthylamine	26	0.30
Mixed exposure	81	1.48

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[a] From Case *et al.*, 1954a,b, with permission.

[b] Expected numbers derived from age-time specific mortality rates for British males.

The excess mortality among persons having had contact with magenta or auramine has been interpreted as indicating that bladder cancer is associated with the manufacture of these chemicals, not necessarily with the substances themselves (Case and Pearson, 1954b). Melick et al. (1971) concluded that 4-aminobiphenyl is carcinogenic in humans from their study that 53 of 315 men exposed to the substance developed bladder cancer.

Studies of persons in the chemical and rubber industries, who were exposed during the manufacture and use of the compounds, have provided evidence that aromatic amines are carcinogenic. Benzidine and naphthylamines are used to manufacture dyes; naphthylamines and 4-aminobiphenyl are antioxidants used to manufacture rubber. Persons who were exposed only to benzidine, 2-naphthylamine, or 4-aminobiphenyl, and who developed bladder cancer can be identified; from this, a causal association between exposure to these chemicals and bladder cancer has been derived.

Another reason the above aromatic amines are accepted as a cause of bladder cancer in humans is that the rate of bladder cancer among those exposed is many times greater than the rate among persons not exposed. Not only is it relatively straightforward to detect the increased cancer incidence among a comparatively small group of exposed persons, it is also clear that apart from the possible effects due to cigarette smoking and certain drugs no other cause of bladder cancer is responsible for the increase. This situation is in contrast to the usual one in epidemiology where, among a group of exposed persons, the rate of the disease of interest is elevated by perhaps only 50-100%. Also, it is often impossible to eliminate or control for the possible effects of other causes of the disease.

Tables 5-2 through 5-5 contain data on mortality and incidence of cancer in the rubber industry from 1940 to 1973. These data were assembled by Monson (1978) from studies of rubber workers in the United States and Great Britain (Andjelkovich et al., 1976; Fox and Collier, 1976; McMichael et al., 1976; Monson and Fine, 1978), who died between 1964 and 1974.

In the United Kingdom, the rubber industry used as a rubber-compounding ingredient a mixture containing a condensate of acetaldehyde with 1-naphthylamine and 2-naphthylamine (Nonox S). The discovery that there was an increased incidence of bladder cancer mortality in those who worked with these compounds (Case and Hosker, 1954) and an associated increased mortality rate in electric cablemakers (Davies, 1965) using contaminated rubber led, in 1949, to the abandonment of the use of Nonox S and other related bladder carcinogens by the rubber industry. Fox and Collier (1976) reported on a survey designed to determine whether the action taken in 1949 had removed the bladder cancer hazard from the industry, but

Table 5-2

Characteristics of Four Groups of Male Rubber Workers[a]

Group	Location	Ethnicity	Number of Workers	Initial Ages	Minimum Years Employed	Years Followed
A	Akron	All	6,678	40-84	10	1964-72
B	Great Britain	All	40,867	35-65	1	1968-74
C	Akron	White	13,571	20-79	5	1940-74
D	Akron	White	8,418	40-84	10	1964-73

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[a] From Monson, 1978, with permission.

Table 5-3

General Mortality Among Four Groups of Male Rubber Workers[a]

Cause of deaths[b]	Group A		Group B		Group C		Group D	
	Obs[c]	Exp[d]	Obs	Exp	Obs	Exp	Obs	Exp
All causes	1873	1798.5	4079	4055.7	5079	6186.9	2373	2524.5
All cancers	351	336.9	1256	1106.0	980	1046.4	457	456.3
Circulatory disease	953	940.0	1999	2022.6	2938	3482.8	1311	1351.7
External causes	59	83.6	118	138.4	278	446.8	91	111.0
Residual	420	438.0	706	788.7	833	1210.9	514	605.5

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[a] From Monson, 1978, with permission.
Expected numbers based on mortality rates for:
A - U.S males
B - English and Welsh males
C - U.S white males
D - U.S white males

[b] Slight differences exist in classification of cause of death.
[c] Observed.
[d] Expected.

Table 5-4

Mortality from Specific Types of Cancer Among Four Groups of Male Rubber Workers[a],[b]

Type of Cancer[d]	Group A		Group B		Group C		Group D	
	Obs.[c]	Exp.[c]	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
Stomach	39	20.9	153	122.3	98	93.9	34	27.6
Large Intestine	39	31.8	e	e	104	103.1	53	45.7
Lung	91	109.3	585	493.5	234	253.1	116	139.8
Prostate	49	34.4	e	e	82	89.0	50	45.9
Bladder	9	12.3	60	38.9	48	39.5	21	18.1
Leukemia	16	12.5	28	23.3	55	43.0	25	18.1

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[a] From Monson, 1978, with permission.

[b] Expected numbers based on mortality rates for:
A - U.S males
B - English and Welsh males
C - U.S. white males
D - U.S. white males

[c] Obs. = Observed
Exp. = Expected

[d] Slight differences exist in classification of type of cancer.

[e] Data not given.

Table 5-5

Observed and Expected Numbers of Bladder Cancers in Selected Work Areas[a]

Group	Work Area	Observed	Expected
A	Receiving and shipping	2	0.7
	Tire building	1	2.5
B	Tires	20	15.3
C	Warehouse/shipping	8	2.6
	Tire building	9	4.8
D	Product fabrication[b]	5	2.6

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[a] From Monson, 1978, with permission.

[b] Product fabrication is subset of tire building.

they refrained from reaching a conclusion because the number of tumors occurring in workers employed after 1949 still had not reached a sufficient level for a statistically valid comparison.

Table 5-3 shows that there is little to suggest a substantial increase in fatal cancer among rubber workers. The greatest increase in bladder cancer has been observed among British rubber workers (Table 5-4). However, compared to the data in Table 5-1, the increment is relatively small. In the U.S. studies, it has not been possible to obtain detailed exposure histories as did Case *et al.*(1954a, 1954b). It was only possible for the investigators to group workers on the basis of where they worked within the factory. The data in Table 5-5 show the observed and expected numbers among men who make tires and who have close contact with uncured rubber. There is minimal evidence that these men have an increased incidence of bladder cancer. Also, among men who make rubber and who might be expected to come into contact with antioxidants such as 2-naphthylamine or phenyl-2-naphthylamine, no increased incidence of fatal bladder cancer was identified.

On balance, there is minimal evidence of an increased incidence of bladder cancer among American rubber workers. Rather than using 1- or 2-naphthylamine as antioxidants, the American rubber industry has used phenyl-2-naphthylamine. Ingestion of phenyl-2-naphthylamine has been shown to be associated with the appearance of minimal amounts of 2-naphthylamine in the urine

(Kummer and Tordoir, 1975). Among an unknown number of men who worked for at least 15 years with a phenyl-2-naphthylamine autoclave, two developed bladder cancer (Monson and Fine, 1978).

HAIR DYES

A number of aromatic amines are used in the manufacture of hair dyes. It has been speculated that users and appliers of hair dyes might be at an increased risk of developing cancer; however, it has not been possible to assess with certainty the effects on humans of specific substances used in hair dye. These are not the only substances to which humans are exposed, and therefore it is not possible to identify persons with isolated exposure. Furthermore, since the adverse effect most postulated is cancer, any substance in hair dyes leading to cancer in either appliers or users will require many years of observation before the effect can be detected. Unless the adverse effect is very strong, it may not be possible to gather sufficient data on which to base an association.

Volume 16 of the *IARC Monographs of the Evaluation of Carcinogenic Risk of Chemicals to Humans* (International Agency for Research on Cancer, 1978b), presents a general review of the data available on the exposures of humans to hair dyes. For users, the results were judged equivocal. There was more evidence for an increased risk of cancer at certain sites (bladder, lung, larnyx) for persons with occupational exposure. However, further epidemiologic studies were recommended before any firm conclusions are drawn.

Reports since that review have not provided more conclusive data. In a followup study of cosmetologists (Walrath, 1978), breast cancer occurred less often than was expected, and leukemia occurred more often than expected. Four recent case-control studies provided minimal evidence for an excess incidence of breast cancer. Crude relative risks between hair dye use and breast cancer were 0.83 (Shore _et al_., 1979), 1.06 (Hennekens _et al_., 1979), 1.11 (Stavraky _et al_., 1979), and 1.28 (Nasca _et al_., 1980). Although stronger associations were observed between subsets of each study group, all authors cautioned against overinterpretation of the results of each individual study.

In summary, the epidemiologic data relating hair dyes and cancer are inconclusive. There is some suggestion that persons with occupational exposure to hair-care products are at increased risk of developing cancer and less evidence that users of hair dyes develop such cancer. These inconclusive studies need to be balanced by the positive results for carcinogenicity and mutagenicity found for a number of the ingredients in hair dyes.

DRUGS

Phenacetin

Among a group of employed Swiss women, those who were regular users of compounds containing phenacetin developed increased serum creatinine levels and low urine specific gravity in comparision to those of controls (Dubach _et al_., 1975; International Agency for Research on Cancer, 1977). Abuse of analgesics has been reported to be associated with the development of renal papillary necrosis (Bengtsson *et al*., 1978). In a number of clinical and epidemiologic studies, heavy users of phenacetin-containing compounds have reported an increased incidence of cancer of the kidney and of the bladder (Fokkens, 1979; International Agency for Research on Cancer, 1977). On the basis of these reports, it seems prudent to associate heavy phenacetin use with kidney and bladder disease in humans.

Chloranaphazine

Chlornaphazine (N,N-bis(2-chloroethyl)-2-naphthylamine), a derivative of 2-naphthylamine, has been used to treat persons with polycythemia and Hodgkin's disease. It is generallly accepted that this antineoplastic agent has led to the development of bladder cancer in humans (Hoover and Fraumeni, 1976; International Agency for Research on Cancer, 1974a; Lower and Bryan, 1979; Thiede and Christensen, 1975; Thiede *et al*., 1964).

Tobacco

Cigarette smoking is the major cause of lung cancer and is associated with the increased incidence of many types of cancer, including bladder cancer (Hammond, 1975). Arylamines and nitrosamines occur in tobacco smoke (Lower and Bryan, 1979). Although the presence of these amines may cause bladder cancer in smokers, the link at the moment is tenuous.

Amitrole (3-amino-1,2,4-triazole)

Amitrole residues were found in cranberries in the United States in 1959. Sale of cranberries and cranberry products from 1958 and 1959 crops were prohibited because amitrole produced thyroid tumors in rats (International Agency for Research on Cancer 1974b).

In Sweden, amitrole was used as an herbicide from the 1950's to the 1970's (Axelson et al., 1974). Increased cancer incidence and mortality rates have been reported among Swedish railway workers who sprayed amitrole and the chlorophenoxyacetic acids (2,4-D and 2,4,5-T) (Axelson et al., 1974, 1979). Among 348 workers, 18 cases of cancer occurred, in comparision to the 11.9 expected from the Swedish incidence rates. Because of the difficulty in separating the possible effects of amitrole and the phenoxy acids, and because many different types of cancer occurred among those exposed, it is difficult to judge whether this excess may be causally related to amitrole exposure.

RECOMMENDATIONS FOR EPIDEMIOLOGIC STUDY OF AROMATIC AMINES

Hair dyes and nitrosamines are the substances that are of most current interest as to their potential carcinogenicity in humans. Nitrosamines are discussed in more detail in a companion report on aliphatic amines. Hair dyes are widely used, and case-control studies have raised suspicions that they are associated with cancer of the breast and other sites. Nitrosamines are recognized to be potent carcinogens in animals and are present in low concentrations in many substances to which humans are exposed. However, there is currently little evidence upon which to judge their carcinogenicity in humans.

The only realistic study design to evaluate the carcinogenicity of these substances is the prospective follow-up study. To study the effects of hair dyes, women should be interviewed to determine their life-time use of hair dyes and followed for 5-20 years to measure the rate of occurrence of cancer. For nitrosamines, persons exposed to relatively high levels in the workplace should be identified, categorized as to level of current (and future) nitrosamine exposure, and followed from 20 to 40 years.

Retrospective studies are not as likely to provide definitive information on carcinogenicity in humans. In case-control studies of cancer, the recollection of hair dye use is subject to a high degree of recall bias. In retrospective cohort studies of persons exposed to nitrosamines, there is a very imprecise measurement of exposure to nitrosamines.

Also, cross-sectional or short-term prospective cohort studies can be conducted on workers exposed to nitrosamines. Here, the health outcome would be either acute illness or physiologic abnormality. These studies would provide an initial evaluation of the association between exposure to nitrosamine and human health. However, they would not be expected to address carcinogenicity.

The epidemiologic evaluation of the possible health effects from exposure to low levels of aromatic amines as well as to other substances may not be possible. To the extent that disease among an exposed group is increased relatively little above background, perhaps less than 50%, the excess may not be detectable against the background variability. One of the best ways to minimize this variability is to conduct prospective follow-up studies, so that at least the measure of exposure is as precise as possible. However, if this strategy is adopted, long-term follow-up is the price that must be paid.

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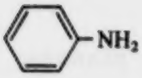
Chapter 6

ANILINE



Aniline (also called aminobenzene or benzenamine) is a colorless, oily liquid that freezes at -6.2°C and boils at 184°C. It is combustible and is moderately soluble in water. At 25°C, aniline has a vapor pressure of 0.67 mm Hg.

Aniline is one of the most important organic bases and is the parent compound for more than 300 chemical products. It is typically produced by the catalytic hydrogenation of nitrobenzene. The gas-phase reaction of hydrogen and nitrobenzene over a catalyst at temperatures below 350°C yields more than 98% aniline.

Aniline as a free base is a relatively unstable compound, which is rapidly oxidized in the presence of air and light to a complex mixture of quinoneimines, quinones, and highly colored polymers of unknown composition. It is a weak base that is readily converted to a water-soluble, stable salt in acid solution (a hydrochloride) (International Agency for Research on Cancer, 1974; Radomski, 1979).

PRODUCTION

Table 6-1 lists the current producers of aniline, their locations, and their annual capacities.

Three of the companies are planning to increase production in the near future. Rubicon Chemicals, Inc. plans to expand its capacity at Geismer, La. by an additional 9,100 metric tons per year during 1980 (Chemical Marketing Reporter, 1979). American Cyanamid Co. will increase the capacity of its Willow Island, W. Va. facility to a total of 50,000 metric tons per year during 1980 (Chemical Marketing Reporter, 1979). The Polyurethane Division of Mobay Chemical Corp. in New Martinsville, W. Va., plans to begin recovery of aniline from its iron oxide plant in the first quarter of 1981. Capacity will be 12,000 metric tons. By 1985, Mobay's polyurethane capacity is expected to reach 18,000 metric tons (Chemical Economics Handbook, 1978).

USES

U.S. consumption patterns of aniline in 1979 are shown in Table 6-2.

Table 6-1

ANILINE PRODUCERS AND CAPACITIES[a]

Producer and Location	Capacity (10^3 metric tons)
Rubicon Chemicals, Inc. Geismar, La.	127
E. I. du Pont de Nemours & Co., Inc. Beaumont, Tex.	118
Gibbstown, N.J.	73
First Chemical Corp., subsidiary of First Mississippi Corp. Pascagoula, Miss.	114
American Cyanamid Co. Bound Brook, N.J.	27
Organic Chemicals Division Willow Island, W. Va.	23
Mobay Chemical Corp., Industrial Chemicals Division New Martinsville, W. Va.	45

Total annual U. S. aniline production for recent years:[b]

Thousands of metric tons			
1975	1976	1977	1978
247.2	265.5	275.4	309.7

[a] SRI (Standard Research Institute), 1979.
[b] U.S. International Trade Commission, 1976, 1977, 1978, 1979.

Table 6-2

ANILINE CONSUMPTION PATTERNS[a],[b]

Use	Percent of total	10^3 metric tons
Intermediate for monomeric and polymeric isocyanates	50	155
Intermediate for rubber chemicals	27	84
Dyes and dye intermediates	6	19
Hydroquinone	5	15
Intermediate for pharmaceuticals	3	9
Miscellaneous	9	28

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[a] Chemical Marketing Reporter, 1979.

[b] Total U. S. consumption is considered equal to U.S production; imports and exports are negligible.

Aniline is used as an intermediate in the production of p,p'-methylenediphenyl diisocyanate (MDI) and polymeric MDI, which are used primarily in the manufacture of rigid polyurethane foam for building insulation (Chemical Economics Handbook, 1978). The U.S. producers of MDI include Mobay Chemical Corp. in Cedar Bayou, Tex., and New Martinsville, W. Va., Rubicon Chemicals, Inc. in Geismar, La., and the Upjohn Co. in La Porte, Tex. (Stanford Research Institute, 1979).

The chemicals derived from aniline are used in rubber manufacture as vulcanization accelerators, antioxidants, and antidegradants (Northcott, 1978). The most commercially significant are 2-mercaptobenzothiazole and N-cyclohexyl-2-benzothiazole (Chemical Economics Handbook, 1978), produced by American Cyanamid Co. in Bound Brook, N. J. the B.F. Goodrich Co. in Henry, Ill., Monsanto Co. in Nitro, W. Va., Pennwalt Corp. in Wyandotte, Mich., and Uniroyal, Inc. in Geismar, La. 2-Mercaptobenzothiazole is also produced by Eastman Kodak Co. in Rochester, N.Y., and the Goodyear Tire and Rubber Co. in Niagara Falls, N.Y. (Stanford Research Institute, 1979).

Dyes prepared from aniline and aniline derivatives are included in the following four dye classes: azo, triphenylmethane, anthraquinone, and safranines (International Agency for Research on Cancer, 1974). The Colour Index (1971) lists 174 dyes that can be prepared from aniline, and more than 700 dyes that can be prepared from aniline derivatives. Because of the increased use of synthetic fibers and stricter controls imposed by the Food and Drug Administration (FDA), very few of these dyes are currently produced in commercially significant quantities (Northcott, 1978). One of the more significant of the aniline-based dyes, from a commercial standpoint is C.I. (Color Index) Vat Blue 1, used widely to dye cotton fibers such as those used in denim. C. I. Vat Blue 1 (D&C Blue No. 6) has also been used as a colorant for surgical sutures. (Bauer, 1979; 21 CFR 74). This dye is produced by BASF Wyandotte Corp. in Parsippany, N. J., and Buffalo Color Corp. in Buffalo, N.Y. (Standford Research Institute, 1979).

Among the commercially more significant dye intermediates derived from aniline are p-nitroaniline, which is produced by Monsanto Co. in Sauget, Ill., American Color & Chemical Corp. in Lock Haven Pa., and the Signal Companies Inc. in Shreveport, La.; N,N-diethylaniline[1] and N,N-dimethylaniline, both produced by American Cyanamid Co. in Bound Brook, N.J., Buffalo Color Corp. in Buffalo, N.Y., and E.I. du Pont de Nemours & Co. in Deepwater, N.Y.; and o-, m-, and p-chloroaniline, which is produced by E. I. du Pont de Nemours & Co., Inc. in Deepwater, N. J. o- and p-Chloroaniline are also produced by Monsanto Co.in Luling, La. (Stanford Research Institute, 1979; Colour Index, 1971).

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[1] Also used to make 2-chloro-2',6'-diethyl-N-(methoxymethyl) acetanilide, an herbicide marketed under the trade name Lasso (Chemical Economics Handbook, 1978).

Aniline is also involved in the production of hydroquinone, which is used primarily as a developing agent for black-and-white photography (Woodlief, 1973), and as a polymerization shortstop in styrene-butadiene rubber production (Bauer, 1979). Hydroquinone is produced by Eastman Kodak Co. in Kingsport, Tenn. and the Goodyear Tire & Rubber Co. in Bayport, Tex. (Stanford Research Institute, 1979.

In the pharmaceutical industry, aniline is used in the production of acetanilide, which was once widely included in analgesic and antipyretic formulations: it is currently used as an intermediate in the manufacture of most sulfanilamide drugs (Northcott, 1978). Pharmaceutical aniline is produced by Eastman Kodak Co. in Rochester, N.Y., Merck & Co., Inc. in Albany, Ga., Salisbury Laboratories in Charles City, Iowa, and Syntex Corp. in Newport, Tenn. (Stanford Research Institute, 1979).

There are a number of miscellaneous applications of aniline. It is used in the production of intermediates for herbicides, fungicides, insecticides, animal repellants, and defoliants (Northcott, 1978) and in the production of cyclohexylamine (formerly an intermediate in the manufacture of cyclamate synthetic sweeteners and presently an intermediate in the production of a variety of other chemicals and as a corrosion inhibitor). Cyclohexylamine and its derivatives are produced by Abbott Laboratories in Wichita, Kans., Monsanto Co. in Sauget, Ill., and Virginia Chemicals Inc. in Bucks, Ala. and Portsmouth, Va. (Stanford Research Institute, 1979). Aniline is also used in the production of p,p'-methylenedianiline, an intermediate for the commercial synthesis of a polyamide fiber marketed under the trade name Quiana. The sole producer of Quiana is E.I. du Pont de Nemours & Co., Inc. The monomer is produced at Belle, W. Va., and the polymer is spun into yarn at the plant in Chattanooga, Tenn. (Chemical Economics Handbook, 1977).

EXPOSURE

As demonstrated above, aniline is produced in large quantities and has numerous applications. Although the potential for human exposure is correspondingly large, there are no quantitative estimates of environmental exposures of the general public. Nonetheless, the National Institute on Occupational Safety and Health (NIOSH), based on results of a National Occupational Hazards Survey, has estimated that a potential 1.26 million workers could be exposed to aniline.

Exposure to aniline in the workplace is regulated by the Occupational Safety and Health Administration (OSHA). The health standards for occupational exposure to air contaminants require that an employee's exposure to aniline shall not exceed

5 ppm or 19 mg/m^3 air in any 8-hour workday of a 40-hour workweek (Occupational Safety and Health Administration, 1980).

In 1979, the American Conference of Governmental Industrial Hygienists (ACGIH) adopted a threshold limit value time weighted average for dermal exposure to aniline and its homologs of 2 ppm or 10 mg/m^3 air for any 8-hour workday or 40-hour workweek; and a threshold limit value, short-term exposure limit of 5 ppm or 20 mg/m^3 air for a period of up to 15 minutes, not to occur more than 4 times per day (American Conference of Governmental Industrial Hygienists, 1979).

Because of aniline's widespread use, it is generally considered to be a likely component of many industrial wastewater discharges. However, the committee found only one reference (Jungclaus et al., 1978) in which aniline concentrations had actually been measured in such a discharge; the aniline concentration in the wastewater discharge. These investigators reported that of a specialty chemicals plant was 0.02 ppm. The compound was not detected downstream of the plant nor in the stream sediment.

Aniline is biodegradable. It is susceptible to treatment in wastewater with activated sludge (Joel and Grady, 1977). In air, it is subject to attack by the hydroxyl radical (Spicer et al., 1974), but its overall half-life in air is not known. No information could be found on the presence of aniline in consumer products, and no evidence was found that aniline is covered by Food and Drug Administration regulations.

The Interagency Testing Committee, established under section 4(e) of the Toxic Substance Control Act (TSCA), has added aniline to its Priority List of Chemicals despite the previous National Cancer Institute (NCI, 1978) test. Chemicals on this list are considered for testing by the U.S. Environmental Protection Agency (EPA) in accordance with section 4(a) of TSCA. Within 12 months of such a recommendation, the EPA must initiate rulemaking to require testing of chemical or publish its reasons for not doing so. The committee recommended studies to determine the carcinogenicity, mutagenicity, teratogenicity, chronic effects, environmental effects, and epidemiology of aniline.

ANALYTIC METHODS

In addition to the general analytic procedures for primary aromatic amines, discussed in Chapter 1, the following additional information from recent literature should help in methods selection.

Iodination and Bromination

The sensitivity of aniline in electron capture-gas chromatography (EC-GC) assays is greatly enhanced by iodination or bromination of the molecule. Kofman *et al*. (1979), described the following process: For iodination, the compound in 1 N hydrochloric acid is treated with sodium nitrite at 0°C, iodinated with potassium iodide at room temperature, and boiled at reflux. The iodine derivative is extracted with hexane; iodination efficiency is 87%. Bromination of aniline is carried out in 1 M sulfuric acid with mesidine, potassium bromide, and potassium bromate. The reaction product (2,4,6-tribromoaniline) is extracted with toluene after alkalinization with 10 N sodium hydroxide. Efficiency of bromination is 99.6%.

Cigarette Smoke

The amines from cigarette smoke were trapped in dilute hydrochloric acid and enriched together with the basic portions, derivatized to pentafluoropropionamides, and determined by EC-GC with a nickel-63 electron-capture detector. The detection limit was approximately 50 pg of aniline per cigarette (Patrianakos and Hoffmann, 1979).

Aniline and its Metabolites

Sternson and Dewitte (1977) reported a high-pressure liquid chromatography (HPLC) method for determining nanomole quantities of aniline and its metabolites, *o*- and *p*-aminophenol, phenylhydroxylamine, nitrosobenzene, nitrobenzene, azobenzene and azoxybenzene, which form nonenzymatically by condensation of reactive metabolites. The compounds were separated by reverse-phase HPLC on a Bondapak carbon-18 column and detected spectrophotometrically. The eluent for the first four components was methanol-water (15:85) containing 0.26 M ammonium acetate and 0.015-M nickel acetate. The remaining components were eluted with a solution of methanol and water (50:50).

Air and Personal Sampling

Wood and Anderson (1975) described procedures for assaying airborne vapors of aniline and related compounds. The vapors were absorbed on silica gel, eluted from the gel with 95% ethyl alcohol containing 0.1% heptanol, and separated and analyzed by gas chromatography with a column of OV-25. Bovkun *et al*. (1974) devised a simple but sensitive and selective method for determining aniline vapors in air. The air sample was drawn through an indicator tube filled with porcelain powder treated with a mixture of alcohol ammonium hexanitrocerate (IV) solution and aqueous potassium persulfate solution. The concentration of aniline in the air was determined by the length of the indicator mass, which had changed colors. Gromiec and Adamlak-Ziemba (1974) determined vapors of N-ethylaniline and aniline in admixture in air. Both compounds were adsorbed in 2% ethyl alcohol, and the sum of the two amines was determined colorimetrically by using an indophenol procedure. Simultaneously, aniline was determined by diazotization and coupling with N-1-naphthylethylenediamine. The concentration of N-ethylaniline was calculated by difference. The determinable limit for N-ethylaniline and aniline was 1.0 and 0.37 mg/ml, respectively.

A rather unique method for personal monitoring was reported by Schaffernicht and Schreinicke (1974). A personal sampler connected to a telemetric system was placed directly on a workman, thereby permitting continuous measurement of the toxic substances in his breathing zone. The toxic substances were colorimetrically determined by absorption in a tube of the personal sampler. Electrolytic current was proportional to the concentration of the toxic substance and was used as the basis for a frequency-modulated telemetric signal. After demodulation on the receiving side, the data were recorded by a strip recorder. The system is suitable for measuring sulfur dioxide, hydrogen sulfide, hydrogen cyanide, phenol, and aniline in the ranges likely to occur in industrial situations. The person being monitored is free to move within a radius of 150 meters around the receiver.

Volumetric Analysis

Madraimov *et al*. (1973) reported an iodometric method for determining aniline or Anesthesin (ethylene aminobenzoate) using a neutral iodine-bromide solution. The procedure is reported to be sensitive to 5 µg of aniline in 0.0005% solutions and 40 µg of Anesthesin in 0.0025% solutions. The preparations were treated with 5 to 6 ml of 1-M hydrochloric acid and 50 ml of 0.1-M iodine-bromine solution and heated at 40°C to 50°C for 3 minutes. After the addition of both 10 ml of ethanol and 10% potassium iodide solution, the released iodine was titrated with a sodium thiosulfate solution, with starch as an indicator.

Other Analytic Methods for Aniline

Ascik *et al*. (1975) determined toxic compounds in pulp and paper mills. They discussed methods of sampling and instrumental analysis and tabulted maximum permissible concentrations for several compounds, including aniline. Zaugol'nikov *et al*. (1975) determined several environmental contaminants, including aniline, and used nomograms and equations to determine maximum permissible concentrations of the compounds in industrial environments, city air, and municipal water reservoirs. Hartstein and Forshey (1974) reported on experiments performed by the Bureau of Mines to investigate products formed on thermal oxidative degradation of selected compounds under both dynamic and static conditions. Four broad classes of materials were studied: polyvinyl chloride compounds, neoprene compounds, rigid urethane foams, and variously treated woods. Thermogravimetric and differential thermal analyses were performed to explore the feasibility of using these analyses to identify materials. Sixteen toxic products including aniline were detected and measured. Dutkiewicz and Szymanska (1973) employed thin-layer chromatography (TLC) to analyze the urine from rats given an oral dose of hydrazobenzene (200-400 mg/kg). Aniline was one of the products detected in the urine.

HEALTH EFFECTS

The primary exposure of humans to aniline is occupational, however, exposure to aniline in the environment may also occur. Aniline is a volatile liquid at room temperature and is rapidly absorbed when inhaled (Dutkiewicz and Piotrowski, 1961; Vasilenko *et al.*, 1972). It is also rapidly and efficiently absorbed through the skin and from the gastrointestinal tract following oral ingestion. These properties led to the establishment of a threshold limit value (TLV) of 5 ppm (19 mg/m^3).

Metabolism

The metabolism of aniline is complex and multifaceted. As with many other compounds, the metabolic process seems to take place in two stages. The first stage, which is mediated by microsomal enzymes in the liver, consists of oxidation (hydroxylation) of the 2 and 4 positions on the aromatic ring and the nitrogen atom (*N*-hydroxylation). Usually 4-hydroxylation predominates with the formation of *p*-aminophenol, the principal metabolite (Parke, 1960; Smith and Williams, 1949; Williams, 1959). *N*-hydroxylation results in the formation of a possible biologically significant metabolite, phenylhydroxylamine. However, phenylhydroxylamine does not appear to be carcinogenic or mutagenic under test conditions where a series of other arylhydroxylamines were positive for both effects (Belman *et al.*, 1968)

The second stage consists of conjugation of these ring hydroxyl groups with glucuronic and/or sulfuric acid. In addition, the unoxidized amine group may be conjugated with glucuronic acid with the formation of a N-glucuronide or with sulfuric acid with the formation of an N-sulfate (sulfamate) (Boyland et al., 1957). Phenylhydroxylamine may react with cysteine, leading eventually to the formation of mercapturic acid conjugates (Boyland et al., 1963)

In all species except dogs, aniline is N-acetylated with the subsequent formation of a second series of metabolites containing the acetyl group (Williams, 1959). Since deacetylation also occurs, these acetyl metabolites are usually present in small quantities in urine. N-oxidation of the acetamide could result in the formation of an hydroxamic acid, N-hydroxyacetanilide, but evidence for its actual occurrence in tissues and urine is lacking. p-Hydroxylation forms N-acetyl-p-aminophenol (Williams, 1959).

Not all of these metabolites occur in all species, and the relative amounts formed vary considerably among species (Conney and Levin, 1974). However, it appears that p-aminophenol is excreted in the urine of all species as a glucuronic acid or sulfate conjugate (Gut and Becker, 1975; Williams, 1959). In rats, 42.3% of the administered dose was recovered as p-aminophenol in urine after acid hydrolysis (Bus et al., 1978) Concentrations of p-aminophenol in the urine of workers has been measured as a rough means of estimating occupational exposure. An average of 39.44 mg/l was associated with the occurrence of significant methemoglobinemia

(Pacseri, 1961). Phenylhydroxylamine is apparently also an important metabolite in most species including humans, but its presence seems to be limited to the blood where it reacts with hemoglobin to form methemoglobin, after which it is oxidized to nitrosobenzene. It has never been detected in the urine of animals given aniline (Kiese, 1966).

Both N-hydroxylation and ring hydroxylation are carried out by the mixed-function oxidase system of the liver microsomes and are stimulated by pretreatment of rat with either phenobarbital or aniline itself and inhibited by SKF 525A (Boobis and Powis, 1975; Conney and Levin, 1974; Patterson and Roberts, 1971; Wisniewska-Knypl and Jablonska, 1975 Wisniewska-Knypl *et al*., 1975;). Aniline is very rapidly metabolized in rabbits and mice; its metabolic half-life in these species is approximately 40 minutes. It is metabolized less rapidly in rats and still less rapidly in dogs (Conney and Levin, 1974).

Acute Toxicity

Mechanism of Methemoglobin Induction. Because of the widespread use of aniline in industry and its high vapor pressure, the occurrence of methemoglobinemia in chemical workers is a rather common experience. The mechanism of the induction of methemoglobinemia by aniline has been widely studied. The bulk of the available evidence indicates that aniline itself is not directly responsible for the induction of methemoglobinemia, but its metabolite, phenylhydroxylamine, is responsible for this effect (Kiese, 1966; Lin and Wu, 1973; McLean et al., 1969;). The phenylhydroxylamine, in the presence of oxygen, reacts with hemoglobin forming methemoglobin and nitrosobenzene. Nitrosobenzene is in turn reduced by the diaphorase nicotinamide-adenine dinucleotide phosphate (NADP)-methemoglobin reductase (in the presence of NADP) back to phenylhydroxylamine, which can in turn oxidize another molecule of hemoglobin. This cyclic reaction goes on until as many as 50 mM of methemoglobin are produced from a single millimole of hydroxylamine (Kiese, 1966). It appears that a small amount of nitrosobenzene is reduced all the way to the amine, and the reaction is then terminated. In addition, nitrosobenzene appears to be inactivated by glutathione, which is present in red blood cells (Aikawa et al., 1978; Eyer,1979;).

NADP-methemoglobin reductase is the enzyme mainly responsible for the physiologic reduction of methemoglobin to hemoglobin. However, NADP-methemoglobin reductase has a greater affinity for nitrosobenzene than for methemoglobin. This affinity inhibits the reconversion of methemoglobin back to hemoglobin as long as the nitroso compound is present.

Sensitivity to the induction of methemoglobin by aromatic amines varies among species. On a mg/kg basis cats are the most sensitive, and humans are approximately 60% as sensitive; dogs are about 30% as sensitive, rats 5%, and rabbits and monkeys seem to be quite resistant to aromatic-amine-induced methemoglobin (Hamblin, 1963). Other evidence indicates that humans are roughly 70 times more sensitive than rats to aniline itself (Jenkins et al., 1972).

Although it seems clear that phenylhydroxylamine is the primary metabolite of aniline responsible for methemoglobin induction, it is not necessarily the only one. For years, p-aminophenol was regarded as the precursor of methemoglobin formation, until it was found that phenylhydroxylamine is approximately 20 times more potent. However, it is possible that p-aminophenol, o-aminophenol, and even other metabolites of aniline may be involved (Smith et al., 1967). p-Aminophenol also requires oxygen to oxidize hemoglobin to methemoglobin, resulting in the formation of the p-quinoneimine, which may go back to p-aminophenol in a manner analogous to the phenylhydroxylamine-nitrosobenzene cycle. However, only a few equivalents of methemoglobin are produced by one equivalent of p-aminophenol (Kiese, 1966).

Although NADP-methemoglobin reductase is normally responsible for the reduction of methemoglobin to hemoglobin, some individuals have a hereditary reduction or absence of this enzyme. Such individuals are hypersensitive to the induction of methemoglobinemia by nitrates. This genetic conditon is due to an autosomal recessive allele and is manifested in homozygotes of both sexes (Goldstein et al., 1968). Such individuals are presumed to be more sensitive to aniline and related methemoglobin inducers, but there is no conclusive evidence for this hypothesis at the present time. Although lack of the reductase may retard the conversion of methemoglobin back to hemoglobin, it may also prevent the reduction of the nitroso compounds back to the hydroxylamine (Radomski, 1979). In contrast, the action of nitrite is a direct one, not involving NADP-methemoglobin reductase.

Although generally regarded as a highly toxic compound for humans, the acute toxicity in laboratory animals is relatively low. The oral LD50's in rats, mice, and cats are 440, 460, and 1,750 mg/kg, respectively. Dermal LD50's are 1,400 mg/kg in rats and 1,290 mg/kg in guinea pigs (National Institute for Occupational Safety and Health, 1977). Fatal poisoning in humans rarely occurs, even following severe exposure. The unfavorable reputation of aniline as an intoxicant is undoubtedly due to the rapidity and efficiency of its absorption through both the skin and the respiratory tract, resulting in the rapid induction of methemoglobinemia. This condition results in symptoms such as headache, nausea, and dizziness (Hamlin, 1963). Indeed, four cases of methemoglobinemia, in which methemoglobin values reached 17-26 g%, were reported from the wearing of shoes dyed black with aniline (Ghiringheili and Molina, 1951).

Methemoglobinemia is a relatively benign and reversible condition, however, at least in normal individuals. Conversion of 75% of the hemoglobin in the body to methemoglobin can occur without life-threatening results (Hamlin, 1963). Experiments in dogs indicates that 4-aminobiphenyl is 10 to 20 times more potent than is aniline, even in the induction of methemoglobinemia. Experiments in cats have shown that p-nitrosobenzene is 50 to 80 times more potent than aniline (Radomski, 1979).

Chronic Toxicity

Carcinogenicity

When Rehn first observed the induction of bladder cancer in chemical workers, he believed these tumors to be due to exposure to aniline and named them "aniline tumors". This unjustified misnomer persisted for many years until a careful epidemiologic investigation by Case *et al*. (1954) and Case and Pearson (1954) convincingly attributed these tumors to 2-naphthylamine and to benzidine, rather than to aniline.

Following these observations, aniline has been regarded as a noncarcinogenic substance. Unfortunately, aniline was never adequately tested in dogs, a test species often used for the evaluation of bladder carcinogens. In the only dog experiment reported in the literature, in which aniline was administered daily to three dogs for 4 years no tumors were observed (Gerhman *et al*., 1948).

Aniline, as the hydrochloride, was given to rats in drinking water in an amount calculated to provide a dose of 22 mg/day for 750 days. One-half of the rats survived longer than 425 days. No tumors of the bladder, spleen, liver, or kidney were observed (Druckrey, 1950). Aniline was also tested both as the free base in lard (1 mg/mouse) or olive oil (8 x 5 mg/mouse) or as hydrochloride in water (13 x 4 mg/mouse). These experiments indicated that aniline was not tumorigenic, when injected subcutaneously into mice in tests ranging from 12 to 24 months (International Agency for Research on Cancer, 1974).

A subacute pilot study in rats was conducted primarily to determine the maximally tolerated dose (MTD) for a chronic feeding experiment. In this experiment, concentrations of 30, 100, 300, and 1,000 mg/kg body weight per day were administered in the diet to male and female Fisher-344 rats. Only the 1,000 mg/kg dose was clearly toxic, causing death in many female rats. In addition, the investigator observed depression of body weight gain, pathology of the liver, kidney, and spleen, and elevated methemoglobin concentrations (Gralla, 1977).

Until recently, these inadequate experiments plus the publication of a series of epidemiologic studies that exonerated aniline as a bladder carcinogen have led to the belief that aniline is not a carcinogen. In 1978, the results of a carcinogenesis bioassay in rats and mice given aniline hydrochloride were released. The compound was fed in the diet of male and female rats and mice for 103 weeks at two concentrations: 0.6% and 0.3% for rats and 1.2% and 0.6% for mice.

Male rats had a significant number of hemangiosarcomas of the spleen. A significant increase in the combined incidence of fibrosarcomas and sarcomas of the spleen and other organs was also observed in rats of both sexes. There was no evidence of aniline-induced carcinogenicity in mice of either sex (National Cancer Institute, 1978). This carcinogenesis bioassay was conducted according to the usual National Cancer Institute (NCI) protocol, which utilizes an MTD and one-half MTD as the doses for the study.

Mutagenicity

The data from mutagenicity tests of aniline are summarized in Table 6-3. Aniline and aniline-derivatives (hydroxylamine and the nitroso derivatives) did not induce mutations in the *Salmonella* test system in four out of five studies, and weakly mutagenic in one (Mitchell, 1978). However, aniline was, in the presence of the "comutagens" norharman and harman (β-carboline derivatives),

TABLE 6-3

Mutagenicity Tests of Aniline

Observations	Species/Strain	Results	References
Gene mutations			
	Salmonella, TA 1535, TA 1537, TA 98, TA 100	neg[a]	McCann *et al.*, 1975
	Salmonella, TA 100, TA 98	neg[a]	Hecht *et al.*, 1979
	Salmonella, TA 1538	neg[a]	Garner *et al.*, 1977
	Salmonella, TA 98, TA 100	pos[b]	Mitchell, 1978
	Salmonella, TA 98	pos/neg[c]	Nagao *et al.*, 1977
DNA damage Mammalian cells in culture	Chinese hamster, V79 cells	neg[d]	Swenberg *et al.*, 1976

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[a] Negative, both with and without metabolic activation system (S-9, microsomes etc.)
[b] Weakly positive in the liquid-medium assay plus the metabolic activation system.
[c] Positive only in the presence of harman or norharman and S-9 fraction.
[d] The V79 cells were incubated with liver S-9 fraction from rat and mouse.

strongly mutagenic in the Salmonella strain TA 98 (Nagao et al., 1977). Aniline caused no DNA strand breakage in mammalian cells in culture (Swenberg et al., 1976). The results are discussed below.

Bacterial Tests. In an extensive study of the mutagenicity of chemical carcinogens in the Salmonella/microsome mutagenicity test, McCann et al. (1975) found aniline to be nonmutagenic in all four tester strains (TA 1535, TA 1537, TA 98, TA 100). Similarly, Hecht et al. (1979), testing aniline as well as the hydroxylamine and nitroso-derivatives, and Garner and Nutman (1977) also obtained negative results for aniline in the Salmonella test. However, Mitchell (1978) obtained weakly positive data on aniline mutagenicity in the Salmonella test by using a liquid-medium method, which involves a liquid incubation of the tester strain, metabolic activation system (S-9 or microsomes) and the test compound before plating the mixture on the agar. Under these conditions, the highest rate of mutagenicity observed was twice the number of spontaneous mutations. The significance of these results is highly questionable: no data were provided on the purity of the compound; no dose-response relationship was observed for the mutagenicity of aniline; the mutagenic effect was independent of metabolic activation and the possibility that the aniline was oxidized under the test conditions to form a mutagenic oxidation - coupling product has not been ruled out.

The nonmutagenic β-carboline derivative norharman has been shown to enhance the mutagenic activity of benzo(a)pyrene, dimethylaminoazobenzene, and tryptophan pyrolysates on *Salmonella typhimurium* strain TA 98 (Sugimura *et al*., 1977). The mutagenicity of aniline and *o*-toluidine was first demonstrated in the *Salmonella* system in the presence of norharman and S-9 fraction from rat liver (Nagao *et al*., 1977). The mutagenicity of aniline followed a clear dose response pattern in strain TA 98, when coincubated with norharman (200 µg/plate). The metabolic changes (activation) of aniline and/or norharman were necessary to demonstrate the mutagenicity of aniline in the presence of norharman, because no mutagenicity was observed without the S-9 fraction (Nagao *et al*., 1977).

Mammalian Cell. Swenberg *et al*. (1976) evaluated the capacity of aniline to induce DNA strand breaks using an *in-vitro*/alkaline elution assay using Chinese hamster V79 cells with and without a liver microsomal activation system. No detectable chromosomal damage, measured as an increase in the rate of DNA elution was observed with aniline.

Teratogenicity

No data were available to evaluate the potential teratogenicity or reproductive toxicity of aniline.

CONCLUSIONS

Until the recent publication of the results of the NCI bioassay of aniline, aniline had been considered noncarcinogenic. Concern about exposure to aromatic amines has always been focused on the induction of bladder cancer because this is the only form of cancer known to have been produced in humans by some of these substances. There was no evidence of the induction of bladder tumors in either the rats or mice at the MTD in the NCI bioassay experiment.

Whether the splenic tumors observed in rats, but not in mice, at the large doses tested in this experiment indicate that aniline represents a carcinogenic threat to humans at a site other than the bladder cannot presently be ascertained. There is no evidence that primary splenic tumors result from the exposure of humans to aniline. On the other hand, there is evidence that a carcinogen may induce tumors in different tissues in different species, and this observation has been extended to the generalization that a substance inducing cancer in any tissue of one species may induce tumors in a different tissue in other species, including humans. Although this may be true as a generalization, there are undoubtedly exceptions since some types of tumors in test animals do not appear to be correlated with carcinogenic potential in humans.

The present TLV for aniline in the United States is in line with that of most other countries, except the Soviet Union, which has lowered it to 0.1 mg/m^3. The basis for this action is unclear (Bardodej, 1975; Vasilenko, 1972; Winell, 1975).

The potential mutagenic activity of aniline has been evaluated extensively in the Salmonella test. The resulting data indicate strongly that aniline alone is without mutagenic effect, although one study reports that aniline is weakly mutagenic (Mitchell, 1978). However, in the presence of the "comutagen" norharman, aniline becomes highly mutagenic in the Salmonella test. However, when aniline and norharman were given in the diet either alone or in combination to male Wistar rats there was no carcinogenic effect to the urinary bladder or other organs which could be treatment related. Although this experiment was terminated after only 80 weeks it does strongly suggest that norharman does not enhance aniline carcinogenicity as it does mutagenicity (Hagiwara et al., 1980).

RECOMMENDATIONS

Although considerable research has already been performed on aniline, gaps in our knowledge of its possible health effects continue to exist, and a compound of such industrial importance deserves to be more thoroughly studied. The carcinogenic effects (hemangiosarcomas of the spleen and sarcomas of the spleen and other organs) observed at the MTD in the NCI bioassay need to be studied further with another lifetime feeding study at 3 or 4 dose levels in a different strain of rat. This will greatly assist in interpreting the significance of the previously observed effects.

A carcinogenicity study using Syrian golden hamsters may also be useful since these animals have been shown to develop bladder tumors after exposure to other aromatic amines. In addition, a long-term (preferably 8 - 10 years), feeding study at the MTD should be conducted on a large number of dogs. Such an experiment will allay any suspicions concerning the possible role of aniline in the causation of human bladder cancer. This test is desirable because the only test with dogs was conducted many years ago on a few animals for too short a time. Along with these studies, there should be further studies on the occurrence of metabolites of aniline in urine to explain the failure of this compound to induce bladder cancer in dogs (if this failure is confirmed). Special attention should be paid to N-hydroxylated urinary metabolites. In addition, studies on the potential for teratogenicity and reproductive toxicity need to be performed.

Further epidemiologic investigations on workers exposed to aniline are also needed. Urine should be monitored and analyzed for aniline metabolites to confirm and quantitate exposures.

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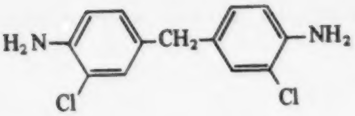
Chapter 7

4,4'-METHYLENE-BIS(2-CHLOROANILINE)



4,4'-Methylene-bis(2-chloroaniline), commonly referred to as MOCA (a registered trade name), is a nearly odorless, crystalline solid that is yellow to tan in color (du Pont, 1977). Its molecular weight is 267, its specific gravity (solid) is 1.44, its melting point is 110°C, and its solubility (% by weight) at 24°C is as follows: trichloroethylene, 4.2; toluene, 7.5; ethoxyethylacetate, 34.4; mesityloxide, 43.0; methylethylketone, 51.0; tetrahydrofuran, 55.5; dimethylformamide, 61.7; and dimethylsulfoxide, 75.0. Its vapor pressure ranges from 1.3×10^{-5} mm Hg at 60°C to 5.4×10^{-5} mm Hg at 120°C.

MOCA is also known by the following synomyms, acronyms, and trade names: diamino-3-chlorophenylmethane, bisamine, di-(4-amino-3-chlorophenyl)methane, 4,4'-diamino-3,3'-dichlorodiphenylmethane, 3,3'-dichloro-4-4'-diaminodiphenylmethane, methylene-bis(o-chloroaniline), p,p'-methylene-bis(o-chloroaniline), DACPM, MBOCA, MCA, Curaline M, Curene 442, and Cyanaset.

PRODUCTION

The sole U.S. producer of MOCA is Anderson Development Company in Adrian, Mich. (Stanford Research Institute International, 1979), but production has presumably been halted as a result of current litigation (see below). du Pont had produced MOCA at its Deepwater, N.J. plant until 1978, when the company decided to phase out production (Chemical Week, 1978).

Because of the proprietary nature of the data, actual production levels are not reported, and estimates of annual MOCA production have varied substantially since 1974 (Table 7-1). As indicated, estimated annual production rates have been as low as 500 kg to more than 4,500 kg. Because estimated annual consumption levels are much higher than these figures, it is likely that production levels are closer to 2 to 3 million kilograms. Some unknown quantity of MOCA is probably imported.

USES

MOCA is applied principally as a curing agent for polyurethanes and epoxy resins which are then used in the manufacture of specialized products, particularly integral-skin polyurethane semirigid foam (used for crash padding) and solid urethane rubber molding such as gear blanks and industrial tires (National Institute for Occupational Safety and Health, 1978). MOCA is added to vary the hardness, flexibility, and impact strength of these products. The most recent information on MOCA consumption indicates that more than 99% is used to manufacture polyurethanes.

TABLE 7-1

Estimated[a] Production of MOCA, 1972-1978

Year	Estimated Gross Production (Thousands of Kg)	Data Source
1972	3,300 approx.	Bell, 1973
1974	0.5	U.S. International Trade Commission, 1975
1975	1+	U.S. International Trade Commission, 1976
1976	4.5	U.S. International Trade Commission, 1977
1977	2,000-2,700[b]	Chemical and Engineering News, 1978
1978	4.5	U.S. International Trade Commision, 1979

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[a] Actual rates are not reported because of company confidentiality.

[b] U.S. consumption.

Polyurethanes cured with MOCA have been used in hundreds of applications (International Agency for Research on Cancer, 1974). Military applications include ball seals on nuclear submarines, positioning strips in Poseidon missiles, and in encapsulation of electric components. In the automotive industry, they have been used in dashboard padding and in numerous small parts. Other reported uses include shoe soles; rolls for postage stamp machines; cutting bars in plywood manufacture; rolls and belt drives on cameras, computers, and reproducing equipment; and wheels and pulleys for escalators and elevators.

Although systems to produce polyurethane elastomers without MOCA have been developed in recent years, many manufacturers continue to use MOCA-based methods because of the superior performance of the resulting products (Ulrich, 1978).

EXPOSURE

The great potential for the distribution of aromatic amines throughout the environment as a consequence of their production has recently been documented (Williams, 1979). Beginning in 1970, a small chemical plant began producing MOCA in the southeastern Michigan town of Adrian. Between 1971 and 1978, production ranged from 184,137 kg to 580,684 kg per year (Harger, 1979). Initially, wastes from the plant were discharged into the Raisin River, which serves as a water source for some downstream communities. After 1973, the wastes were channeled into a lagoon before they entered Adrian's wastewater treatment system. In the winter of 1978-1979, continuing problems at the treatment facility prompted a closer examination of the materials coming from this lagoon and the plant. Local residents had long been disturbed by odors and dust that periodically permeated the area. Consequently, when the state recognized that MOCA was one of several products of this plant, numerous samples were analyzed.

The results of these studies disclosed that nearly 18 km^2 is contaminated with MOCA including approximately one-half of the town of approximately 20,000 inhabitants. The compound appears to have been spread by every possible mechanism. Airborne particulates were the probable source of the material (up to 400 ppm) that collected in the eavetroughs of adjacent houses (Michigan Department of Natural Resources, 1979). Mechanical tracking from the plant was suggested by the high levels of MOCA along the road that led from the facility. Surface soil samples from public roads as far as 1.6 km from the plant contained up to 2 ppm MOCA; those adjacent to the plant, up to 590 ppm; garden and yard samples from the local residences contained, up to 55 ppm; and house dust from vacuum cleaners, up to 18 ppm (Michigan Department of Natural Resources, 1979). According to Parris et al. (1980) and Walkington (1979), transport via water was evident from the MOCA content of sludge from the settling lagoon (1,600 ppm), the wastewater treatment plant (18 ppm) ,and the Raisin River (10 ppm). Secondary transport of the compound also is believed to have occurred as a consequence of the agricultural use of the sludge from the wastewater treatment plant.

Evidence that humans have been exposed is provided by analyzing urine for MOCA. There are no methods for anzlyzing urine for its metabolites. In experiments with rats, only a small quantity (usually less than 0.3%) of MOCA was excreted unchanged (Kommineni _et al_. 1978). Thus, the actual exposure is far greater than is implied by the concentration detected in the urine. Since essentially nothing is known about the metabolic disposition of MOCA in animals or humans, it is difficult to estimate exposure from urine analyses. Furthermore, it is likely that there is significant excretion of MOCA in bile. Available MOCA metabolism data indicate that the substance behaves as a polymorphic substrate for the acetyl CoA-dependent _N_-acetyltransferase of both rabbit and human liver (Glowinski _et al_., 1978). The genetically determined levels of this enzyme may, in fact, determine the rapidity with which MOCA is excreted and/or converted to the reactive species involved in the carcinogenic process. From previous knowledge of aromatic amine metabolism _in vivo_, it is also expected that MOCA metabolites would be excreted relatively rapidly. Thus far, only the 5-hydroxy derivative has been reported as a urinary metabolite from an observation made in a study of dogs, a species incapable of _N_-acetylation (Barnes, 1964). The paucity of knowledge about MOCA metabolism complicates attempts to evaluate exposure by analyses of urinary excretion.

In spite of these complications, however, three categories of individuals were shown to have been exposed: the workers, their families (including spouses and children of all ages), and preschool children (ages 2 to 5 years) living in the area of the plant

(Williams, 1979). It is believed that the families were contaminated by workers carrying MOCA home on their clothing since direct contamination of clothing was demonstrated. The preschool children were presumed to have been exposed while playing in MOCA-contaminated soil; older neighborhood children did not have detectable levels of MOCA (i.e., < 0.3 ppb) in urine. The workers were found to have up to 59 ppm MOCA in their urine; the members of the workers' families had urine MOCA levels of up to 15 ppb; neighborhood children had up to 2 ppb. The detection of MOCA in the urine of the workers' families was not dependent on the location of residence.

It is not known whether plant materials grown in the gardens of the area represent a source of human exposure. Experiments to resolve this question are in progress. Measurments suggest that the level of MOCA in the soil has declined little if any since the summer of 1979.

Analyses of urine specimens from workers at several facilities that used the product from Adrian and other sources demonstrated that some individuals excreted up to 0.7 ppm MOCA (Harger and Saftlas, 1979). These observations confirm findings of earlier studies (Hosein and Van Roosmalen, 1978). Environmental samples obtained from sites that had not been exposed to MOCA for more than 4 years still contained detectable levels of the amine (Schleusener, personal communication, 1980). As far as is known, the Adrian plant has been the only MOCA production site in the United States for several years. Since current litigation has presumably halted production in Adrian, MOCA is now believed to be available to American users only from foreign sources. Conceivably, the use of MOCA-containing products could be a hazard. It is possible, for example, that hydrolysis of MOCA-containing polyurethanes could release very low levels free MOCA, or unreacted MOCA might be leached from plastic under certain conditions (Henning, 1974).

Occupational exposures to MOCA are of concern, as indicated by recent federal actions. In 1969, the Food and Drug Administration disallowed the use of MOCA as a component of certain food-contact articles (Federal Register, 1969). The National Institute for Occupational Safety and Health (NIOSH) recommended a standard of 3.0 $\mu g/m^3$ in breathing zone air determined as a time-weighted average. The Occupational Safety and Health Administration (OSHA) is expected to set a standard for MOCA in 1980. The American Conference of Governmental Industrial Hygienists (ACGIH) has adopted a threshold limit value, time-weighted average concentration of 0.02 ppm for MOCA (American Conference of Governmental Industrial Hygienists, 1979).

A national NIOSH survey (1978) indicated that in the early 1970's approximately 55,000 U.S. workers could have been exposed to MOCA. The majority of these workers were employed in small- to medium-sized establishments.

Concentrations in the workplace were sampled in one study in Italy. As indicated in Table 7-2, concentrations ranged from 0.04 to 4.5 mg/m^3--much higher than the proposed OSHA standard. It is not possible to judge the similarity between Italian and U.S. production conditions.

Table 7-2

Sampled Concentrations of MOCA in the Workplace at One Facility in Italy[a]

Sample Location	Duration (minutes)	MOCA Concentration mg/m^3)
Near the blending reactor where MOCA is mixed manually	180	0.0400
Near another reactor where MOCA is mixed automatically	180	0.110
Above the oven in which the container of MOCA is reheated	140	0.283
Near the oven in which the container of MOCA is reheated	140	0.041
Above the exhaust of the MOCA blending reactors	140	4.5

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[a] Source: Abstracted from Traina *et al*., 1978.

ANALYTIC METHODS

In additon to the general procedures already described for primary aromatic amines, most of the analytic methods for MOCA have focused on the analysis of air or urine by using gas chromatography (GC) or high-pressure liquid chromatography (HPLC) procedures.

Sawicki (1975) sampled air by drawing it through a tube of Gas-chrom S. Sections of the tube packing were then extracted with 0.5 ml of acetone and analyzed by direct injection into a gas chromatograph equipped with a 30-cm long, 2.3 mm (internal diameter) stainless steel column packed with 10% Dexsil 300 GC coated on 80/90 mesh Anakrom ABS. The investigator used helium as the carrier gas and a flame ionization detector. An injection of 1 μl of the acetone extract permitted the detection of 2 ng of MOCA or approximately 2 $\mu g/m^3$ for a 500-l air sample. In field trials, no impurity was encountered that caused interference with the retention time of MOCA. Isomers of chloroaniline commonly associated with MOCA were completely resolved and did not interfere. The solvent effect was pronounced in that the MOCA peak appeared on the tailing edge of the acetone peak. It was therefore necessary to restrict injection volumes to 2 μl or less. At about the same time, Yasuda (1975) reported a method essentially identical to that of Sawicki, except that a 0.33 m long, 0.04 cm (internal diameter) stainless steel column packed with the 10% Dexsil 300 GC was used. Sensitivities of both methods were identical.

Van Roosmalen et al. (1979) reported a procedure to determine trace levels of MOCA in urine. The samples were partially cleaned up by solvent extraction, followed by thin-layer chromatography (TLC) on plates of silica gel G. The MOCA was then extracted from the TLC plate, converted to its trifluoroacetyl derivative, and analyzed by GC. The gas chromatograph was equipped with a 1.8 m long, 0.32 cm (internal diameter) glass column packed with 3% OV-1 on Gas Chrom Q and a flame ionization detector (FID). A detection limit of 1 μg/l was claimed. After the investigators prepared the trifluoroacetyl derivative which is highly electron-capturing, they chose to use the FID and did not mention the possibility of using the more sensitive and specific electron capture-gas chromatograph system.

The only HPLC procedure found in the literature for MOCA was reported in 1979 by Rappaport and Morales (1979) for determining airborne exposure of humans to MOCA. A personal sampler consisted of a filter to remove the particulate MOCA, followed by a bed of silica gel to remove the vapors. The compound was extracted from the sampler stages with methanol, and a 10 μl aliquot was injected into an HPLC instrument equipped with a reverse-phase system and a 254 nm UV detector. Quantitation of 3 ng of MOCA corresponded to 0.15 μg/sample. Precision levels were 9.2% and 14% for 1.5 and 0.15 μg samples, respectively.

Metabolism

Although little is known about the disposition of MOCA, it is likely that it is metabolized via the pathways have been demonstrated for other aromatic amines. These pathways include *N*-oxidation, *N*-acetylation, C-oxidation, and conjugation with glucuronate or sulfate as described elsewhere. Indirect evidence for the *N*-oxidation of MOCA comes from its mutagenicity in a *Salmonella* assay (see mutagenicity section). The genetically polymorphic acetyl CoA-dependent acetylation of MOCA has been demonstrated with preparations from both human and rabbit liver (Glowinski *et al*., 1978). Evidence for the oxidation of the aromatic ring is supported by the report that 5-hydroxy-MOCA is a urinary metabolite of the parent amine in dogs (Barnes, 1964).

By analogy to other aromatic amines, transformation of MOCA to derivatives capable of reacting with tissue macromolecules can be expected to occur as a consequence of the production of an *N*-oxidized derivative and a subsequent activation step. With other compounds, this latter step has been shown to involve the formation of reactive *N*-sulfoxy and/or *N*-acetoxy metabolites. An alternative activation pathway can result from the peroxidation of the amine. MOCA possesses one structural feature, the methylene bridge carbon, not usually found in aromatic amines. This feature may make possible an additional pathway for metabolic activation. Conjugates of metabolites oxidized at the methylene carbon may, for example, be reactive as a consequence of this benzylic structure. If this type of metabolite were to be responsible, in part, for the carcinogenicity of MOCA, studies predicated solely on MOCA's aromatic amine structure might fail to detect the involvement of this kind of metabolite.

Acute Toxicity

The LD_{50} (single dose) of MOCA in rats (strain and sex unspecified) is 750 mg/kg (Barnes, 1964). As with most other aromatic amines, MOCA produces methemoglobin in rats and dogs. Rabbits appear to be less susceptible to the acute toxic affects of MOCA: the acute lethal dose by skin application was < 5 g/kg. The protection afforded rabbits by their effective N-acetylation of MOCA may reduce methemoglobin formation as compared to its formation in rats, which are less effective acetylators of aromatic amines, or dogs, which are unable to acetylate aromatic amines. Although du Pont has reported no acute toxicity in humans as a consequence of its manufacture of MOCA (Linch *et al*., 1971), an increased urinary frequency and hematuria (Mastromatteo, 1965) and a transitory inability to reabsorb low molecular weight protein and concentrate urine (Hosein and Van Roosmalen, 1978) have been related to MOCA exposure.

Chronic Toxicity

Carcinogenicity

MOCA is of primary concern because of its demonstrated carcinogenicity in animals, and its structural relationship to aromatic amines known to be capable of inducing bladder cancer in humans. It is carcinogenic in mice, rats, and dogs (Table 7-3).The evidence for tumor development is less strong in mice (Russfield et al., 1975). Only hepatoma development in female CD-1 mice was significantly greater in the treated animals. Two dose levels, 1,000 and 2,000 ppm, were used, but the effective number of animals, 21 and 14 per group, respectively, were small. The length of the experiment, 24 months, was appropriate, and the animal survival adequate. There was a suggestion, however, that a greater number of animals would have resulted in a significant increase of vascular tumor induction.

Experiments proved that MOCA does induce bladder tumors in dogs (Stula et al., 1977). Doses of 8 to 15 mg/kg/day were given 5 times per week for up to 9 years. Five of six treated dogs developed transitional cell carcinomas of the lower urinary tract. None of the six control dogs developed these tumors. Thus, the carcinogenicity of MOCA in the bladder of dogs is roughly comparable to that of 2-acetylaminofluorene and 3,3'-dichlorobenzidine, with respect to dose and total test period; somewhat less carcinogenic than 2-naphthylamine; much less carcinogenic than 4-aminobiphenyl; and more carcinogenic than benzidine (Table 7-4).

TABLE 7-3

Tumor Induction by MOCA

Tumor	Species Mouse	 Rat	 Dog
Lung adenocarcinoma		+	
Mammary adenocarcinoma		+	
Zymbal gland adenocarcinoma		+	
Hepatocellular carcinoma		+	
Hepatoma	+	+	
Hemangiosarcoma		+	
Bladder			+

TABLE 7-4

Comparative Carcinogenicity of (Orally Administered) Aromatic Amines in the Lower Urinary Tract of Dog[a]

Compound	Total Intake Per Dog (Mol)	Treatment Period (Yr)	Total Period (Yr)	Tumor Incidence
Benzidine	1.77	5.0	7.0 - 10.0	3/7
MOCA	0.77 - 0.85	8.3 - 9.0	8.3 - 9.0	5/6
3,3'-Dichlorobenzidine	0.65 - 0.70	6.6 - 7.1	6.6 - 7.1	4/5
2-Acetylaminofluorene	0.20 - 0.89	2.6 - 3.9	5.7 - 7.5	4/5
2-Naphthylamine	0.24 - 0.28	2.6	2.6	4/5
4-Aminobiphenyl	0.03 - 0.04	2.8 - 3.1	2.8 - 3.1	6/6

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[a] Adapted from Stula *et al.*, 1977.

The most detailed data on the carcinogenicity of MOCA are available on male rats (Kommineni _et al._, 1978). Three dose levels of MOCA were given to male CD-rats, fed either a protein-adequate or protein-deficient diet. Sufficient numbers of animals were used for each group (50 to 100 per group), and the animals were observed for a long period. The mean survival time ranged from 65 to 89 weeks. A summary of the pertinent data are presented in Table 7-5. Kommineni _et al._ have appropriately pointed out that the urine levels of MOCA in these animals is actually less than in some of the exposed workers. It seems very likely that, if a similar experiment were to be conducted with female rats, the mammary incidence of mammary gland tumors would be significantly higher than it was in the male animals used in this experiment. Although Stula _et al._ (1975) used female animals in the earlier study, the differences in survival between control and treated animals clouded the interpretation of the results.

The only information regarding the carcinogenicity of MOCA in humans is du Pont's report (Linch _et al._, 1971) of its limited experience producing the compound. The authors indicated that they had not observed malignancies attributable to MOCA in 31 men with exposure lasting 6 months to 16 years.

Mutagenicity

MOCA has been tested for mutagenicity in a large number of systems, but the only results reported thus far are in the

TABLE 7-5

Incidence (Percentage) of Predominant Neoplasms in Male Rats Fed MOCA[a]

	Protein-Adequate Diet				Protein-Deficient Diet			
MOCA (ppm)	0	250	500	1,000	0	125	250	500
No. of rats autopsied	100	100	75	50	100	100	75	50
Lung adenocarcinomas[b]	0	14[c]	27[c]	62[c]	0	3	9[d]	16[c]
Mammary adenocarcinomas	1	5	11[d]	28[c]	0	1	4	6[e]
Zymbal gland carcinomas	1	8[e]	7	22[c]	0	0	5[e]	12[c]
Hepatocellular carcinomas	0	3	4	36[c]	0	0	0	18[c]
Hemangiosarcomas	2	4	4	0	1	2	5	8[b]
Mean survival (weeks)	88.9	86.6	80.4[d]	65.3[c]	87.3	80.6	79.4	76.8[e]
Urinary MOCA (ppb) at 26 weeks	<10	30	70	330	<10	<10	60	120

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[a] Abstracted from Kommineni *et al*., 1979, with permission.

[b] Includes bronchiolar-alveolar cell carcinomas.

[c] Difference from respective controls is significant ($p \le 0.001$).

[d] Difference from respective controls is significant ($p \le 0.01$ and >0.001).

[e] Difference from respective controls is significant ($p \le 0.05$ and > 0.01).

Salmonella/microsome test. These reports either include no data (Ho *et al.*; 1979; Takemura and Shimizu, 1978) or have reported the results obtained as bacterial revertants per single dose (Anderson and Styles, 1978; McCann *et al.*, 1975).

McCann *et al.* (1975) reported MOCA as positive in the TA 100 strain of *Salmonella* with Aroclor-induced rat liver S-9 (1,050 revertants at the 100 µg/plate dose, equivalent to 2.7 revertants/nmol). Anderson and Styles (1978) reported a fivefold increase over background at 100 µg/plate in the TA 100 strain using rat liver S-9. Ho *et al.* (1979) also indicated that MOCA was mutagenic in yeast, but reported no information as to strain, test system, or treatment.

MOCA was also tested for mutagenicity as an unknown in 37 laboratories in a variety of test systems in the International Program for Evaluation of Short Term Tests for Carcinogenicity (IPESTTC). The data from this study have not yet been published. However, MOCA was positive in bacterial DNA repair tests, phage induction tests, the *Salmonella*/microsome tests, the *Salmonella* fluctuation test, and in *E. coli* WP-2. Mixed results were observed in the seven yeast systems used. MOCA induced unscheduled DNA synthesis in HeLa cells in culture but did not induce sister chromatid exchanges (SCE's) in culture. It produced transformation in baby syrian hamster kidney (BHK) cells in culture. *In vivo*, MOCA produced sex-linked recessive lethal mutations in *Drosophila melanogaster* and mixed results in the micronucleus test in rodents. Further analysis of these data must await the report's publication.

MOCA was also tested in a preincubation modification of the Salmonella/microsome test in the National Institute of Environmental Health Sciences (NIEHS) Environmental Mutagenesis Test Development Program. S-9 from Aroclor 1254 induced male rat and Syrian hamster liver was used with strains TA 98, TA 100, TA 1535, and TA 1537. MOCA was mutagenic for strains TA 98 and TA 100 with S-9; hamster S-9 produced a higher response than rat S-9 in the TA 100 strain, and the responses in TA 98 were equivalent (K. Mortlemans, personal communication).

Teratogenicity

No data were available to evaluate the potential teratogenicity or reproductive toxicity of MOCA.

CONCLUSIONS

MOCA is a mutagen for Salmonella, requiring liver S-9 for its activity. Results in press show it is also active in bacterial repair tests, phage induction tests, E. coli mutagenesis, Drosophila, and cell transformation in vivo. Mixed results were observed with yeast, chromosomal effects in cultured cells, and the micronucleus test in vivo. A more complete evaluation awaits publication of the IPESTTC study.

Studies in test animals have demonstrated conclusively that MOCA is a carcinogen. This activity is to be expected from its structure, which is similar to that of other aromatic amines that induce tumors in humans as well as in animals. Although the paucity of epidemiologic evidence does not permit an evaluation of the carcinogenic effects of MOCA, it is reasonable to assume that, given a sufficiently high exposure, it may also be carcinogenic in humans.

RECOMMENDATIONS

The MOCA-exposed population in and around Adrian, Mich. and individuals exposed as a consequence of the use of MOCA should be studied further to learn whether or not the compound is carcinogenic in humans. This goal requires three types of effort. The first is to explore the metabolic disposition of MOCA so that methods for evaluating exposure to it can be developed. The second phase is to apply these methods, including an evaluation of necropsy specimens from any member of this population who dies during the course of investigation. Such studies would help better define the potential for risk to individuals, as well as aid in monitoring the effects of the cleanup efforts. The final step is the prospective surveillance of this population to determine whether exposure to MOCA increases their tendency to develop cancer.

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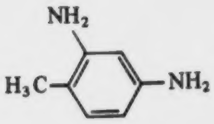
Chapter 8

2,4-DIAMINOTOLUENE



2,4-Diaminotoluene (toluene-2,4-diamine; 2,4-DT) is a colorless crystal that melts at 99°C. It is soluble in hot water.

2,4-DT synthesis takes place in three steps, beginning with the nitration of toluene in a mixture of nitric and sulfuric acids at 30°C to 70°C. The resultant mononitrotoluene mixture is then nitrated again in a somewhat stronger acid medium to a mixture of dinitrotoluene isomers, of which the largest fraction is 2,4-dinitrotoluene (75.8%). A number of processes can then be used to produce 2,4-DT from 2,4-dinitrotoluene. All of these methods involve catalytic hydrogenation, followed by purification, to remove unwanted isomers and byproducts.

PRODUCTION

Table 8-1 lists the current producers of 2,4-DT, their locations, and (where available) their estimated annual capacities (Chemical Economics Handbook, 1977-1978; Stanford Research Institute International 1979; United States International Trade Commission, 1979).

TABLE 8-1

Producers of 2,4-DT

Company and Plant Location	Estimated Annual Capacity as of January 1, 1979[a] Thousands of metric tons
Air Products and Chemicals, Inc.	
Industrial Gases Div.	
Middlesex, N. J.	NR[b]
Pasadena, Tex.	57[a]
Allied Chemical Corporation	
Specialty Chemicals Division	
Moundsville, W. Va.	30
American Cyanamid Co.	
Organic Chemicals Division	
Bound Brook, N. J.	NR[b]
BASF Wyandotte Corporation	
Polymers Group	
Urethane Division	
Geismar, La.	36
E. I. du Pont de Nemours & Company, Inc.	
Elastomer Chemicals Department	
Deepwater, N. J.	25
Mobay Chemical Corporation	
(owned by Bayer AG, Federal Rep. of Germany)	
Polyurethane Division	
Cedar Bayou, Tex.	47
New Martinsville, W. Va.	36
Olin Corporation	
Olins Chemicals Group	
Ashtabula, Ohio	11
Brandenburg, Ky.	NR[b]
Lake Charles, La.	36
Rochester, N. Y.	NR[b]
Rubicon Chemicals Inc.	
(jointly owned by Uniroyal, Inc., and Imperial Chemical Industries Limited, United Kingdom)	
Geismar, La.	15

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[a] Estimates of production capacities are based on an assumed 2,4-diaminotoluene capacity of 0.8 metric tons for each metric ton of toluene diisocyanate capacity, except for Air Products and Chemicals, Inc., which does not produce toluene diisocyanate.

[b] Not reported.

Total U.S. production of 2,4-DT has declined in recent years (U.S. International Trade Commission, 1976-1978) as shown in Table 8-2.

USES

2,4-DT is used almost exclusively for the production of toluene diisocyanate. Other U.S. consumption is estimated to amount to less than 230 metric tons annually (Chemical Economics Handbook, 1977-1978).

Mixtures of isomers of 2,4-DT are normally used to produce toluene diisocyanate. The most important mixture contains 80% of the 2,4-isomer and 20% of the 2,6-isomer. Eight U.S. companies produced 284,072.4 metric tons of the 80/20 toluene diisocyanate mixture in 1978 (International Trade Commission, 1978). A mixture containing 65% 2,4-isomer and 35% 2,6-isomer is also used in significant quantities. Only a small quantity of 2,4-DT is isolated for conversion to pure 2,4-toluene diisocyanate.

The Chemical Economics Handbook (1977-1978) estimated the U.S. consumption pattern for toluene diisocyanate in 1978 as shown in Table 8-3.

2,4-DT can be used to produce (approximately) 60 dyes, which are used to color silk, wool, paper, boat fibers, cellulosic fibers, and cotton. The following nine dyes are believed to have been produced from 2,4-DT in the United States during 1978: Basic Brown 4, Basic

Table 8-2

Annual Production of 2,4-DT[a]

Year	Thousands of metric tons
1976	105.9
1977	101.1
1978	63.3

[a] Data from U.S. International Trade Commission, 1976-1978.

Table 8-3

U.S. Consumption of Toluene Di-isocyanate in 1978[a]

Use	Percent of Total
Flexible polyurethane foams	83%
Polyurethane surface coatings	6
Polyurethane elastomers	3
Other (including rigid polyurethane foam)	8
Total	100%

[a] Data from Chemical Economics Handbook, 1977-1978.

Orange 1, Direct Brown 2, Direct Brown 154, Direct Black 4, Leuco Sulphur Orange 1, Leuco Sulphur Brown 10, Solvent Brown 12, and Sulphur Black (Colour Index, 1975; U.S. International Trade Commission, 1978). They are used in spirit varnishes and wood stains as indicators, in the manufacture of pigments, and as biologic stains.

2,4-DT is used as a developer for direct dyes, particularly to obtain black, dark blue, and brown shades, and to obtain navy blue and black colors on leather. It is also used to dye furs and was an ingredient in hair dye formulations until banned in 1971 (International Agency for Research on Cancer, 1978).

EXPOSURE

More than 99% of the 2,4-DT produced in the United States is used to produce toluene diisocyanate, generally at the site of production. The single exception is the Air Products and Chemicals Plant at Pasadena, Tex., which produces no toluene diisocyanate and, conversely, a 45,000 metric ton/year capacity toluene diisocyanate plant at Freeport, Tex. operated by Dow Chemical, which does not produce its own 2,4-DT. Therefore, the main sources from which humans are exposed will be the plants listed in Table 8-1. However, there is no information on which to base an analysis of either occupational or general exposures from plant emissions.

Because the substance is solid at ambient temperatures, air emissions from 2,4-DT production do not appear to be significant.

However, contamination of wastewater discharges from the plants is a likely route of exposure. The hydrogenation of dinitrotoluene yields 4 mol of water for every mol of 2,4-DT produced (600 l of water per metric ton of 2,4-DT). This water is separated in a dehydration column at 100°C-150°C at atmospheric pressure (Milligan and Gilbert, 1978). Because the vapor pressure of 2,4-DT is 11 mm Hg at 150°C and it is soluble in hot water, the separated water is undoubtedly highly contaminated. This wastewater is probably treated before it is discharged.

Some 2,4-DT could find its way into consumer products as an impurity in dyes. However, such exposure is nearly impossible to quantify.

Under the U.S. Food and Drug Administration (FDA) regulation concerning 2,4-DT, (21 CFR 177) is listed under the category of antioxidants and antiozonants. The total of these components is not to exceed 5% by weight of rubber product.

The U.S. Occupational Safety and Health Administration does not have an occupational standard covering the exposure of workers to 2,4-DT.

ANALYTIC METHODS

Jones et al. (1978) reported separations of nondye components in the commercial food color preparation Brown FK. The dye, which is manufactured by the reaction of diazotized sulfanilic acid with a mixture of m-phenylenediamine and 2,4-diaminotoluene, has been shown to consist of six major colored components. High-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC) procedures were evaluated for their ability to separate a mixture of the six dye components as well as the starting compounds (e.g., 2,4-DT) used in the synthesis. Two different columns and solvent systems were used in the HPLC investigations with a UV absorption detector set at 254 nm. One of the systems, which consisted of a column of Partisil 5 loaded with 7% aminopropyl phase, was subjected to a 30-minute linear gradient from acetonitrile-water (2:3) to acetonitrile-water (2:3 containing sodium biphosphate (2 g/l). Only partial resolution of the components was obtained. The other HPLC system, which consisted of a column of Chromosorb Si 100 loaded with a 21% octadecyl phase subjected to a 17-minute linear gradient from 5% to 40% acetonitrile in water (containing 1.2 and 2.4 g/l of sodium biphosphate and sodium hypophosphate, respectively) was more successful: all nine components of the mixture were separated with essentially baseline resolution.

Unger and Friedman (1979) developed an HPLC procedure to assay 2,6- and 2,4-DT. Their technique was adaptable to biomonitoring and metabolic studies with samples of urine and plasma from rats. A normal-phase silica column was used with a mobile phase consisting of acetonitrile-water saturated chloroform (8:2) and a 250 nm UV absorption detector. The two compounds were resolved as sharp peaks in 3 minutes, and from 1 ng to 2 ng of each substance was quantitated. Dichloromethane extraction of urine and plasma spiked with 2,4-DT (10 to 200 ppm), yielded recoveries of about 90% or more.

TLC tests were performed by using unactivated silica gel G plates (layer thickness 0.25 mm) with a solvent system of phenol-water (4:1). The starting materials of the manufacturing process (e.g., 2,4-DT) were detected by spraying with 1% Ehrlich's reagent in 50% acetic acid. Although the TLC system resolved for the six dye components, it was not suitable for the other compounds since sulfanilic acid was not resolved from one of the dye components. The two aromatic amines (2,6- and 2,4-DT) were not well resolved and ran close to the solvent front.

Spectrophotofluorimetry (SPF) was the basis for a method described by Guthrie and Mckinney (1977) to analyze 2,4- and 2,6-DT in flexible polyurethane foams at levels as low as 1 ppm. The amines were extracted with methanol, separated by TLC, and assayed by SPF after reaction with Fluram reagent. The SPF assay was accomplished by uniformly spraying the developed and dried TLC plate with an 0.015% solution of Fluram in acetone and measuring the fluorescent spots (λ_{EX} = 390, λ_{Em} = 500 nm) with a thin-film chromatographic scanner. The instability of the Fluram derivative required that quantitative measurements be completed within 1 hour. The nonuniform characteristic of foams was determined by assaying sample extracts in duplicate at the 10 to 15 ppm level. Precision, usually within ±10% for a given extract, was ±30% for six different samples of the same foam.

Lepri *et al*. (1976) investigated the behavior of several coloring agents (including 2,4-DT) used in oxidation-type hair dyes on thin layers of various ion exchangers. Some of the systems provided unique separations. Examples of such systems are AG 1-X4 ($CH_3CO_2^-$) developed with 0.1 mol acetate buffer in a 4:1 water-methanol mixture; BD-cellulose developed with 0.5 mol acetate buffer solution; Dowex 50-X4 (H^+) with 0.1 mol acetate buffer in a 1:1 mixture of water-methanol; Rexyn 102 (H^+) developed with various mixtures of dimethylformamide-water; and AG 3-X4A developed with 95% ethyl alcohol. R_f values were tabulated for some 25 compounds.

Two direct gas chromatography (GC) procedures for separation and analysis of isomeric diaminotoluenes were reported in 1968. Willeboordse *et al*. (1968) separated mixtures of 2,3-, 3,4-, 2,4-, 2,5-, and 2,6-DT using a mixed partitioning agent of Carbowax 20M and Saponate DS-10 on base-loaded Chromosorb G, followed by Saponate DS-10 on the same solid support. Boufford (1968) separated a mixture of 3,4-, 2,3-, 2,4-, 2,5-, and 2,6-DT on a column of 5% Bentone 34 plus 155 Hyprose SP-80 [octakis(2-hydroxypropyl)-sucrose] on potassium-hydroxide-treated Chromosorb W at 170°C. Helium carrier gas and a hydrogen flame ionization detector (FID) were used.

The analysis of 2,4-DT was specifically mentioned in several of the procedures described for primary aromatic amines. Additional discussions in the literature (details unavailable) involve in situ TLC determinations of toluenediamine and methylenedianiline isomers in the products of hydrolytic degradation of polyurethanes (Lesiak and Orlikowska, 1978) and an ultrasonic method to assay amine solutions, including toluenediamine (Bogdanova et al., 1976).

HEALTH EFFECTS

Metabolism

2,4-DT is rapidly absorbed after intraperitoneal injection into rats and mice, and peak levels in blood or serum are attained within 1 hour after injection (Grantham *et al.*, 1979). Less than 1.5% of the dose is excreted unchanged. Metabolites are rapidly excreted, predominantly in urine (Grantham *et al.*, 1979; Waring and Pheasant 1976). Unger *et al.* (1980) confirmed these observations in male B6C3F mice administered a single intraperitoneal dose of 2,4-[^{14}C]DT. They noted rapid absorbtion from the peritoneal cavity with the dominant route of excretion via the kidneys; one hour after dosing almost 50% of the radioactivity was recovered in the urine. They also noted that the three tissues which have shown a carcinogenic response to 2,4-DT (hepatocarcinoma, rhabdomyosarcoma, and fibrosarcoma), either acquired an initial high concentration of 2,4-DT (liver) or demonstrated an extended elimination half-life (muscle and skin).

Major metabolic reactions include acetylation of one or both amino groups oxidation of the methyl group to the benzylic alcohol and benzoic acid functions and ring hydroxylation, primarily at the 3 and 5 positions. Glinsukon *et al.* (1975) examined the ability of liver cytosol from various species to N-acetylate 2,4-DT. The liver cytosol from hamsters had the most enzymatic activity followed by guinea-pig, rabbit, mouse and rat. Human liver cytosol formed only trace amounts of acetyl derivatives while dog liver cytosol had no activity. Although the liver cytosol showed the greatest acetylating activity, the cytosol from the kidney, intestinal mucosa, and lung also were able to produce significant amounts of various acetylaminotoluenes. In this study 4-acetylamino-2-aminotoluene was the major metabolite and 2,4-diacetylaminotoluene the minor metabolite. Glucuronide and sulfate conjugation of these primary metabolites also occurs, varying with the species examined. No information is available on the mechanism of metabolic activation of 2,4-DT.

Acute Toxicity

National Institute of Occupational Safety and Health (1979) reported that the oral LDLo (lowest published lethal dose) of 2,4-DT in rats was 500 mg/kg, and that the LDLo's by subcutaneous injection to rats, dogs, and rabbits were 50, 200, and 400 mg/kg, respectively. More recent data on the acute toxicity of 2,4-DT after intraperitoneal injection in to male Fischer rats and female NIH Swiss strain mice indicate LD_{50} values of 325 mg/kg and 480 mg/kg, respectively (Grantham et al., 1979).

Gosselin et al. (1976) reported that no poisonings of humans by 2,4-DT were known. Nonetheless, they rated the compound as very toxic, reporting a probable oral LDLo in humans of 50-500 mg/kg. On the other hand, National Institute for Occupational Safety and Health (1979) lists the oral LDLo of 2,4-DT in humans as 50 mg/kg.

Teratogenicity

No data are available on the embryotoxicity or teratogenicity of 2,4-DT.

Carcinogenicity

Subcutaneous Administration. Twenty rats (mixed strains and sex) were given 0.5 ml of 0.4% solution of 2,4-DT in propylene glycol subcutaneously at weekly intervals for approximately 8 months (Umeda, 1955). The survival rate at 8 months was only 45%. All rats surviving 8 months developed subcutaneous sarcomas. The total dosage of 2,4-DT ranged from 60-90 mg per rat. No contemporary controls were used in this experiment.

Oral Administration. Ito et al. (1969) first reported the carcinogenicity of 2,4-DT after oral administration. They observed carcinoma of the liver in male Wistar rats fed diets containing 600 or 1,000 ppm 2,4-DT for 33 to 34 weeks. Tumor incidences at 36 weeks were 9/9 (100%) after a dosage of 1,000 ppm 2,4-DT and 7/11 (64%) after a dosage of 600 ppm. Histologically, all the liver tumors were hepatocellular carcinomas. Many of the rats also had multiple metastatic tumors in lymph nodes, omentum, lungs, and epididymis. The livers of six control rats fed the basal diet without 2,4-DT were essentially normal. Weisburger et al. (1978) confirmed the hepatocarcinogenicity of 2,4-DT (as the dihydrochloride) after oral administration in CD-1 (Sprague-Dawley) rats and in HaM/lCR mice.

More recently, the National Cancer Institute (1979) selected 2,4-DT for additional bioassay in the Carcinogenesis Testing Program. Groups of 50 F344 rats of each sex received 2,4-DT in feed at two dose levels. Time-weighted average doses were 79 ppm for 103 weeks (low dose) and 171 to 176 ppm for 79 to 84 weeks (high dose). The incidences of hepatocellular carcinomas or neoplastic nodules were dose related (males: controls 0/20, low-dose 5/49, high-dose 10/50; females: controls 0/20, low-dose 0/50, high-dose 6/49). In addition, carcinomas or adenomas of the mammary gland occurred in female rats (controls 1/20, low-dose 38/50, high-dose 41/50). The most common type of mammary tumor by far was the fibroadenoma. Other types of tumors appeared less frequently than did liver or mammary tumors, but were assumed to be related to exposure to 2,4-DT. These included lung tumors, squamous cell carcinoma of the skin and preputial gland, pancreatic acinar cell adenomas, subcutaneous fibromas and fibrosarcomas, and mesotheliomas.

In parallel studies, groups of 50 B6C3F1 mice of each sex received 2,4-DT in feed at two dose levels, either 100 ppm or 200 ppm, for 101 weeks. Hepatocellular carcinomas occurred in female mice (control 0/19, low dose 13/47, high-dose 18/46). In addition, lymphomas occurred at a significant incidence in the low-dose female mice (controls 2/10, low-dose 29/47, high-dose 11/46). No tumors occurred at significantly increased incidences in the dosed male mice.

Skin Application. Two studies (Burnett et al., 1975; Giles et al., 1976) involving the skin painting of mice with hair dye formulations that included 2,4-DT have been performed. In one study (Giles et al., 1976), mice were given weekly topical applications of 0.05 ml of either a 6% solution of 2,4-DT alone or hair dye formulations containing 2,4-DT. The results of this study were judged inadequate (International Agency for Reserarch on Cancer, 1978) for an evaluation of the carcinogenicity of 2,4-DT because a large number of animals were unaccounted for in the final analysis of tumor incidence.

The second study (Burnett et al., 1975) involved the painting of 0.05 ml of a hair dye formulation containing 0.2% of 2,4-DT on the skin of Swiss-Webster mice weekly or fortnightly for 18 months. Carcinogenicity of 2,4-DT could not be evaluated on the basis of this study because of the complexity of the applied mixture and because of the reported high incidence of tumors observed in control mice (International Agency for Research on Cancer, 1978).

Mutagenicity

The data from mutagenicity and other genotoxic tests of 2,4-DT are summarized in Table 8-4. The substance was mutagenic in both the *Salmonella* and *Escherichia coli* systems (Ames et al., 1975; Aune et al., 1979; Pienta et al., 1977; Venitt, 1978) and in *Drosophila melanogaster* (Blijleven, 1977; Fahmy and Fahmy, 1977). Negative results were obtained in *Neurospora crassa* (Ong, 1978) and in the micronucleus test in the rat (Hossack and Richardson, 1977). Potentially genotoxic effects (chromatid and chromosomal gaps and breaks in peripheral lymphocytes) from hair dyes which may have contained 2,4-DT, among other ingrediants, were observed in professional hair colorists and others with a history of hair dye use (Kirkland et al., 1978).

TABLE 8-4

Mutagenicity Tests of 2,4-DT

Observations	Species/Strain	Results	Reference
Gene Mutation			
Bacterial	*Salmonella*, TA 1538, TA 98	pos[a]	Ames *et al*. 1975
	E. coli, 343/113 (arg^+ reversion)	pos[a]	Venitt, 1978
Yeast	*N. crassa*, ad-3A	neg[b]	Ong, 1978
Insect	*D. melanogaster*, Oregon K, Berlin K	pos[c]	Fahmy and Fahmy, 1977 Blijleven, 1977
Chromosome Damage			
Micronucleus test in rat		neg	Hossack and Richardson, 1977
Chromosome damage humans		pos[d]/neg	Kirkland *et al*., 1978

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[a] Mutagenic in the presence of S-9 (metabolic activation).
[b] Tested without metabolic activation.
[c] X-Chromosome recessive (lethals and visibles).
[d] Chromatid and chromosome gaps and breaks.

Bacterial Tests. The studies of Ames *et al*. (1975), Aune *et al*. (1979), and Pienta *et al*. (1977) clearly demonstrate that 2,4-DT is a potent mutagen in the *Salmonella* system. Like many other aromatic amines, 2,4-DT requires metabolic activation (S-9 or microsomal fractions) for mutagenesis. Principally, it causes a frameshift type of change in strains TA 1538 and TA 98. Pienta *et al*. (1977) reported a correlation between the 2,4-DT-induced morphologic transformation in an *in vitro* carcinogenesis system (using secondary culture target cells prepared from cryopreserved, primary Syrian hamster embryo cells) and the mutagenicity of 2,4-DT in the *Salmonella* system. Venitt (1978) reported mutagenicity of 2,4-DT in the *E*. *coli* system.

Yeast. Ong (1978) used the adenine-3 (ad-3) forward-mutation system of *N*. *crassia* to test the mutagenicity of environmental agents and chemical carcinogens, including 2,4-DT. 2,4-DT was nonmutagenic in the *N*. *crassa* system; however, since no mammalian metabolic activation system was included in the test, these data must be regarded as inconclusive.

Insect. Fahmy and Fahmy (1977) examined the comparative mutagenic effect of 2,4-DT as well as that of phenylenediamine and benzidine in *D*. *melanogaster*. The compounds were injected at equimolar dose range (5-20 mM) into the abdomens of adult males, and mutagenicy was measured separately on the various stages of spermatogenesis. Genetic activity was assayed with respect to the overall induction of the X-chromosome recessive (lethals and visibles) mutations relative to the specific effects on ribosomal DNA (bobbed locus). All the compounds exerted mutagenicity, both on the X-chromosome and on the RNA genes, but activity on the different genic sites varied between compounds and as a function of cell stage. The mutagenic potency toward the bobbed locus was benzidine>2,4-DT> 4-*N*-*o*-phenylenediamine, which correlates with the carcinogenic potency of these compounds.

Blijleven (1977) observed similar mutagenicity by feeding 2,4-DT to male *D. melanogaster* and measuring sex-linked recessive lethal mutations. Metabolically active germ cells (spermatids and spermatocytes) had the highest mutagenic activity.

Chromosomal Damage.

Micronucleus Test. Hossack and Richardson (1977) obtained negative results in micronucleus tests of 2,4-DT and 11 other hair dye constituents. Groups of five male and five female rats were gastricly intubated with the test compounds at 0.5% (w/v) in gum tragacanth containing 0.05% sodium sulpfite. The total dosages were close to the lethal doses and were administered in two equal parts, separated by an interval of 24 hours. The animals were killed 6 hours after the second dose and bone-marrow smears were prepared. The incidence of micronucleated cells per 2,000 polychromatic erythrocytes was compared with the values from the control group. The mean values and ranges of micronucleated cells were not significantly different for the control and 2,4-DT-treated groups. Similar results were obtained for the other compounds.

Chromosomal Damage in Humans. No data are available on the possible genotoxic effects of 2,4-DT *per se* in humans. However, in a recent epidemiologic study of the potentially genotoxic effects of hair dyes (Kirkland *et al*., 1978), chromosomal damage was investigated in peripheral-blood lymphocytes of professional hair colorists. The authors found no significant differences in chromosomal damage in cultured peripheral-blood lymphocytes from 60 professional hair colorists as compared with those of 36 control subjects closely matched for age and sex. There was a statistically significant excess of chromosomal damage (mainly chromatid breaks) in women with dyed hair when age-matched women were regrouped according to whether their hair was dyed or not. Men (mean age 22.9 years) with dyed hair had significantly less chromosomal damage than did men (mean age 31.5 years) whose hair was not dyed.

Possible confounding factors in these findings are that most tinters wear gloves when applying hair dyes and, even without gloves, percutaneous absorption of hair dye constituents may be effectively impeded by the horny surface of the hands and by the lack of sebaceous glands in the palms; hair dye constituents are readily absorbed through the scalp, which contains numerous sebaceous glands; and the lower frequency of chromosomal aberrations in young men with dyed hair (compared with the frequency in slightly older men without dyed hair) is probably due to an age effect. This preliminary evidence on the genotoxic effects of hair dyes in humans warrants further study in view of the known mutagenicity and carcinogenicity in animals of several hair dye constituents.

CONCLUSIONS

2,4-DT is carcinogenic in rats and mice after oral administration; the produces amine produces liver and mammary gland tumors. There is some evidence that it also induces sarcomas at the site of subcutaneous injection in rats. Published data on the carcinogenicity of 2,4-DT after application to mouse skin are not adequate for evaluation.

2,4-DT is a potent mutagen in microbial test systems and causes germ cell mutation in *D. melanogaster*. The substance may cause chromosomel damage to (chromatid and chromosome gaps and breaks) in humans. It also induces cell transformation in an *in vitro* mammalian carcinogenesis test system. However, there are no data, such as case reports or epidemiologic studies, on the carcinogenicity of 2,4-DT in humans.

RECOMMENDATIONS

There is absolutely no information available on the mechanism by which 2,4-DT is activated in susceptible species, including rats and mice. However, given the demonstration of carcinogenicity in two species of animals and the data on the genotoxic effects in *in vitro* systems, it is prudent to assume that humans are under some increased risk from exposure to 2,4-DT. Recommendations for future research with 2,4-DT include studies of the mechanism by which the chemical is activated in rats, mice and humans, testing for carcinogenicity in additional species to obtain more data on the relationship between metabolism and carcinogenicity, and examination of the *in vitro* metabolism of 2,4-DT in human tissues to supplement the preliminary observations of Glinsukon *et al*. (1975). This kind of additional data would make it easier to estimate the extent of risk to humans exposed to 2,4-DT.

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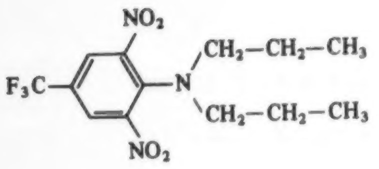
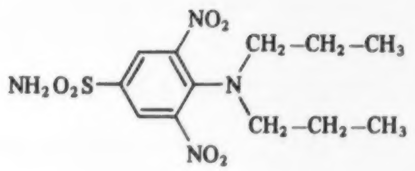
Chapter 9

TRIFLURALIN AND ORYZALIN





Trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine; also known as Treflan) and oryzalin (3,5-dinitro-$\underline{N}^4$, $\underline{N}^4$-dipropylsulfanilamide) are both members of a class of compounds known as dinitroanilines. Trifluralin is an orange crystalline solid that melts at 49°C. It has a low solubility in water (0.3 ppm) and a low vapor pressure (2×10^{-4} mm Hg at 30°C). Oryzalin is a yellow-orange crystalline solid that melts at 141°C. It has a much lower vapor pressure (less than 10^{-7} mm Hg at 30°C) than trifluralin, but is slightly more soluble in water (2.5 ppm at 25°C).

Trifluralin is produced via a series of reactions beginning with the reaction of hydrogen fluoride with *p*-chlorotoluene to produce 4-trifluoromethylchlorobenzene. The latter compound is then nitrated followed by reaction with di-*N*-propylamine, which replaces the chlorine to form trifluralin. The production of oryzalin begins with the nitration of *p*-chlorobenzenesulfonic acid, followed by the addition of chlorine to form 2,6-dinitro-*p*-chlorobenzenesulfonyl chloride, followed by the addition of ammonia and di-*N*-propylamine to yield oryzalin.

PRODUCTION

Eli Lilly and Co. (Elanco Products Division) is the sole U.S. producer of trifluralin (Stanford Research Institute International, 1979; U.S. International Trade Commission, 1978). The plant at Lafayette, Ind., has an estimated 23,000-metric ton capacity for production of trifluralin.

Lilly owns the patent rights for oryzalin, but has contracted production to various other firms since receiving approval of oryzalin use in 1975. GAF Corporation made oryzalin for Lilly at Rensselaer, N.Y., from January 1975 to June 1976 (Chemical Week, 1979). Proctor Chemical at Salisbury, N.C., produced oryzalin from January 1975 to January 1976. The U.S. International Trade Commission listed the Sodyeco Division of Martin Marietta Corporation, Sodyeco, N.C., as a producer of oryzalin in 1977 and 1978. Other processors and formulators of oryzalin for Lilly have included Bold Chemical of Tifton, Ga., Central Chemical of Hagerstown, Ark., and Helena Chemical of Helena, Ark. (Chemical Week, 1979). Although no estimates of production capacity are available for these plants, the production of trifluralin in 1975 and 1978 is estimated at 12,000 and 16,000 metric tons, active ingredient, respectively. Oryzalin production in 1975 is estimated at 45 metric tons, active ingredient; 1978 production is estimated at 450 metric tons of active ingredient.

USES

Trifluralin and oryzalin are both used almost exclusively as herbicides. Consumption estimates for each in 1978 are shown in Table 9-1.

The Environmental Protection Agency (EPA) has approved both trifluralin and oryzalin for use as herbicides; however, both compounds have been challenged on several occasions. Trifluralin was challenged on the grounds that it contained nitrosamine contaminants and that its use therefore posed an unacceptable carcinogenic risk. Risk-benefit studies initiated by EPA concluded that benefits of trifluralin outweigh any risks if nitrosamine contamination is kept below 1 ppm (Chemical Marketing Reporter, 1979). Subsequently, Lilly has produced trifluralin with a nitrosamine content of less than 1 ppm, and has continued to receive approval for its use as an herbicide.

Oryzalin has been suspected of causing heart-related birth defects among children fathered by workers involved in its manufacture and of causing skin rashes in workers. However, EPA investigations did not disclose any adverse effects, and no regulatory action is planned (Chemical Marketing Reporter, 1980). Nonetheless, Lilly is required to maintain ongoing teratological studies to support the continued registration of oryzalin (Pesticide and Toxic Chemical News, 1980).

Table 9-1

Consumption of Trifluralin and Oryzalin

Product Containing Compound	Percent of Total	10^3 Metric Tons of Active Ingredient
Trifluralin		
Cotton	21%	2.7
Deciduous fruits/nuts	1	0.05
Peanuts	1	0.1
Soybeans	74	9.7
Sugar beets	1	0.05
Vegetables	2	0.3
Other field crops	1	0.14
Industrial/commercial	1	0.1
Total	100	13.1
Oryzalin		
Cotton	10	0.05
Deciduous fruits/nuts	10	0.05
Soybeans	60	0.27
Industrial/commercial	20	0.09
Total	100	0.45

Stanford Research Institute, 1979

EXPOSURE

The principal routes of exposure to trifluralin and oryzalin are the following: workers are exposed during manufacturing process; agricultural workers are exposed while applying the substances to crops; and the general public is exposed through releases of the compounds into air and water during manufacturing processes, through drift, volatilization, and runoff as a result of application, and via contaminated food crops. Because more trifluralin is used than oryzalin, and because much more data about trifluralin exist, this chapter focuses on exposure to trifluralin.

Trifluralin is produced at a single site in Lafayette, Ind. (population, 45,000); thus, exposure of the public to trifluralin during its manufacture is of only local importance. Although no data are available on trifluralin plant discharges, small quantities are known to be discharged into the Wabash River as waste from the manufacturing processes. The treatment procedure involves activated sludge, followed by settling, and then filtration through activated carbon (Spacie and Hamelink, 1979).

Parts per trillion (ppt) concentrations of trifluralin have been measured in the water downstream from the plant. However, the tissues of fish captured in these waters contain concentrations of trifluralin several thousand times greater than those in the water (Spacie and Hamelink, 1979).

The most likely source from which the general public is exposed to trifluralin is the herbicide's application to soils to control grass and broadleaf weeds. This is practiced primarily in the North Central and South Central States, especially in Illinois, Iowa, and Mississippi (Helling, 1976).

Trifluralin is not an especially persistent chemical. There have been some studies of the persistence of trifluralin in soil under actual field conditions (Golab _et_ _al_., 1979; Golab and Amundsen, 1975; Probst _et_ _al_., 1967) These studies show that trifluralin concentrations decreased from 10% to 15% of their original value 1 year after application. Trifluralin is lost through volatilization as well as through degradation. At this rate of loss, trifluralin does not tend to accumulate in soils receiving repeated applications.

Although the rate of volatilization of trifluralin depends to a great extent on the method of its application, such dissipation provides a significant potential exposure route for persons living downwind of treated fields. If trifluralin is applied to the soil surface, up to 90% of the compound may be volatilized within 2-3 days of application. If it is incorporated into the soil, volatilization losses can be as little as 3% or 4% in 90 days (Taylor, 1978). In an experiment in which trifluralin was sprayed onto soil and then tilled into the soil, White _et_ _al_. (1977) measured a maximum concentration of 16.5 $\mu g/m^{-3}$ 20 cm above the soil during application. The maximum concentration was 3.4 $\mu g/m^{-3}$ 20 cm above the soil on the second day after the compound was tilled into the soil, decreasing to 100 ng/m^{-3} after the 35th day. They estimated total volatilization losses to be at 22.4% during the growing season (120 days) and vapor losses during spraying to be at 3.5%.

Exposure of persons living downwind of sprayed fields is somewhat reduced because trifluralin is subject to fairly rapid photochemical decomposition. Measurements of trifluralin degradation in air indicate a half-life of 20 minutes under midday summer sunlight conditions. Woodrow et al. (1978) observed that the half-life increased to 193 minutes in the fall. Trifluralin is stable in the dark.

Although relatively immobile in soil, trifluralin may be transported long distances from its initial source of application via runoff into streams and rivers. Indeed, trifluralin has been detected at levels of up to 0.2 μg/l in the Cape Fear River, N.C. Trifluralin concentrations tended to correlate with periods of greatest runoff and soil erosion on agricultural lands adjacent to tributaries of that waterway (Horden, 1977). Although there is probably minimal direct exposure of humans via this route, the tendency of trifluralin to accumulate in fish must not be overlooked (Spacie and Hamelink, 1979).

Humans may also be exposed to trifluralin through food crops treated with the herbicide. However, analyses of residues in a wide variety of tolerant crops indicate that trifluralin is not incorporated into the leaves, seeds, or fruits (measurement sensitivity, 5-10 ppb). In treated root crops, such as onions and garlic, trifluralin residues are found only in the outer shell, which is usually discarded before consumption. The exception is carrots: 31% of the total residue of 0.65 ppm was found in the interior of the carrot proper--69% was concentrated in the peel (Probst *et al*., 1967).

ANALYTIC METHODS

Methods to analyze trifluralin in a variety of vegetables, plant tissues, soil, water, oily crops, wheat grain and straw, and mint and mint hay are described in the Pesticide Analytical Manual of the Food and Drug Administration (1973). Trifluralin is extracted from crops by blending it with methanol and subsequent extraction from the solvent into dichloromethane. After evaporation of the dichloromethane, the extract is dissolved in hexane, cleaned up on a column of Florisil, and analyzed by electron-capture gas chromatography (EC-GC). GC columns (1.83 m) packed with 3% XE-60 or 5% SE-30 on Chromosorb W are used at approximately 180°C with the carrier gas flowing at 90 ml/minute. The retention time (t_R) for trifluralin is 3 to 4 minutes. The analytic procedure is modified as required for aqueous or oily samples. Recoveries of approximately 80% or more can be expected, and the sensitivity ranges from 5 to 10 ppb. Samples containing residues of benzene hexachloride, ethion, and/or zineb require additional cleaning. The interfering compounds are separated by thin-layer chromatography (TLC). The trifluralin is then scraped from the plate, eluted, and assayed by EC-GC.

The procedure used at North Carolina State University (T.J. Sheets, North Carolina State University, personal communication, 1980) to determine trifluralin residues in soils requires a 4-hour Soxhlet extraction of the oven-dried sample with benzene-isopropyl alcohol (1:2). The extract is evaporated just to dryness, dissolved in hexane, cleaned on a Florisil column and subsequently analyzed by EC-GC. Glass columns (1.83 m long 0.635 cm outside diameter) packed with 4% SE-30 plus 6% QF-1 or with 5% Carbowax 20M on Gas Chrom Q are operated at 155°C with a gas flow of 80 to 100 ml/minutes. Under these conditions, the t_R for trifluralin is approximately 2.0 minutes.

Recently reported analytic methods have focused mainly on GC techniques, coupled with various types of detectors including mass spectrometers. Payne et al. (1974) developed a procedure to analyze trifluralin, diphenamid, and paraquat in admixture in soil, sediment, and water. The procedure permits simultaneous GC assays of trifluralin and diphenamid, without cleanup, by employing a Coulson electrolytic conductivity detector. Paraquat is determined colorimetrically. The 1.83 m long glass GC column (0.635 cm outside diameter) was packed with 10% DC-200 on Gas Chrom Q. Recoveries of trifluralin spiked into water at concentrations of 0.05 to 10 ppb were 82% to 91%; those from soil spiked at 0.05 and 1.0 ppm were 86% and 94%.

Smith (1974) used acetonitrile-water (9:1) and ultrasonication to extract residues of four herbicides, including trifluralin, from three types of soil. The addition of excessive amounts of water and saturated sodium sulfate solution to the extract facilitated subsequent partitioning of the herbicide residues into hexane. The hexane extract was then concentrated and assayed by EC-GC. The GC system consisted of a 1.5 m long glass column (6 mm outside diameter), packed with 10% OV-1 on Chromosorb G-HP operated at ml/minute, t_R's for dichlorobenil, trifluralin, dinitramine, and triallate were approximately 1.0, 2.9, 4.5, and 5.0 minutes, respectively. Recoveries of all four compounds spiked into the three types of soil at 0.05 and 0.5 ppm levels were 92% or more.

Lawrence (1976) examined the separation characteristics of several GC liquid phases (i.e., 3% OV-1, 4% SE-30-6% SP-2401, 10% DC-200, and 5% DEGS on Chromosorb W HP) for 12 pesticides, including trifluralin, by using the Coulson detector. Sensitivities (50% scale) for trifluralin at a t_R of approximately 3 minutes on OV-1 at 175°C were reported as 6 ng and 3 ng for the nitrogen and chlorine modes, respectively. Later, Lawrence et al. (1977) reported a confirmatory procedure for pesticides that contain nitrogen dioxide (NO_2). The pesticide residue in 1 ml of benzene was shaken with 0.5 ml of aqueous chromous chloride to convert the NO_2 groups to NH_2. The solution was made basic and the product extracted with benzene for analysis by GC with a Coulson detector (nitrogen mode). The products were found to be approximately as sensitive as the parents and had t_R values of 0.4 to 0.9 minutes relative to the parent compound on the SE-30/SP-2401 column. The procedure was used to confirm trifluralin residues in extracts of potato spiked at levels of 0.5 to 1.0 ppm.

Woodrow et al. (1978) used EC-GC to study the behavior of trifluralin vapors released into the atmosphere as emulsifiable concentrate sprays. The experiment was conducted with a 1.8 m long glass column (3 mm, internal diameter), packed with 3% OV- 17 ng/m^3. In a similar study, Soderquist et al. (1975) studied photoproducts of trifluralin in air by using EC-GC, TLC, and GC-mass spectrometry (MS). The photoproducts were subjected to TLC on 0.5 mm plates of silica gel G containing 1% zinc orthosilicate phosphor and developed with hexane-acetone (3:1). The resulting bands were scraped off, eluted with acetone, and quantitated GC-MS by using a 1.5 m long glass column (3 mm, internal diameter) containing 25 OV-1 on Chromosorb G. The column temperature was programmed to rise from 150°C to 270°C at 10°/minute with a helium carrier flow of 16 ml/minute. As each compound eluted, its mass spectrum was recorded and identified by comparison with an authentic specimen. Samples of analysis by EC-GC were analyzed under conditions similar to those described by Woodrow (1978).

Downer et al. (1976) compared analytical results for residues of benefin and trifluralin in soil by using a GC-MS procedure and an established EC-GC assay. Comparable sensitivities (<10 ppb) and results were obtained for both the mass fragmentography (multi-ion detector, MID) and the EC-GC procedures. The MID procedure was said to have a shorter analysis time and to be less susceptible to contamination than were EC-GC assays.

Heck et al. (1977) reported a high-pressure liquid chromatography (HPLC)-MS procedure developed to obtain pharmacokinetic data from rats dosed with trifluralin. Isotope dilution analysis, with nonfragmenting mass spectrometry offered several advantages over other techniques for determining trace quantities of organic compounds in biologic materials. Residues of the compound were separated from rat tissue and excreta by sequential high-pressure gel permeation and reverse-phase liquid chromatography. The cleaned samples were then quantitated by field ionization MS.

HEALTH EFFECTS

The mutagenicity and chronic toxicity of both trifluralin and a contaminant, N-nitrosodipropylamine (NDPA), have been reviewed extensively by EPA's Special Pesticide Review Division, Office of Pesticide Programs (OPP), and is reported in "Trifluralin (Treflan) Position Document" (Environmental Protection Agency, 1979). A discussion of specific needs of EPA with several representatives from the OPP led to the decision that this committee should focus only on a review of the possible genetic toxicity of trifluralin. Consequently, the committee limited its study to a review of the EPA's documents, which is summarized below with minor editorial changes and updated information from several studies.

Trifluralin Mutagenicity Data

Technical trifluralin, containing known and undetermined levels of NDPA, has been tested in a number of system without producing mutagenic effects. The results of these studies are presented in Tables 9-2 and 9-3.

TABLE 9-2
Mutagenicity Tests of Trifluralin (Part I) and NDPA (Part II)

(Part I)

Test System	Technical Trifluralin		References
	Species/Strain	Result	
Gene Mutations			
Bacterial	*Salmonella typhimurium* (8 Strains)	Neg[a]	Anderson *et al.*, 1972
	Salmonella typhimurium[b,c]		
	TA 100	Neg	Simmon *et al.*, 1977
	TA 1535	Neg	Simmon *et al.*, 1977
	TA1537	Neg	Simmon *et al.*, 1977
	TA1538	Neg	Simmon *et al.*, 1977
	Escherichia coli[b,c]		
	WP2	Neg	Simmon *et al.*, 1977
Insect	*Drosophila melanogaster*	Neg	Murnik, 1978
Other	*Escherichia coli* with T_4 bacteriophage	Neg[a,d]	Anderson *et al.*, 1972
Chromosomal Mutations			
Insect	*Drosophila melanogaster*	Neg[e]	Murnik, 1978
Primary DNA Damage			
DNA repair, bacteria	*Escherichia coli* W3110/p 3478	Neg[b,c]	Simmon *et al.*, 1977
	Bacillus subtilis H17(Rec$^+$)/m^{45}(Rec$^-$)	Neg[b,c]	Simmon *et al.*, 1977
Yeast mitotic recombination and/or gene conversion	*Saccharomyces cerevisiae* D3	Neg[b,c]	Simmon *et al.*, 1977
Mammalian cell Unscheduled DNA synthesis	Human Fibroblasts WI-38 Cells	Neg[b,c]	Simmon *et al.*, 1977

TABLE 9-2

Mutagenicity Tests of Trifluralin (Part I) and NDPA (Part II)
(Part II)

Test System	Species/Strain	NDPA Result	NDPA References
Gene Mutations			
Bacterial	*Salmonella typhimurium*		McCann *et al.*, 1975
	Unspecified	Pos[f]	Yahagi *et al.*, 1977
	TA 98	Neg[f]	Yahagi *et al.*, 1977
	TA 100	Pos[f]	Yahagi *et al.*, 1977
	TA 1530	Pos[f]	Bartsch *et al.*, 1976 Camus *et al.*, 1976
	TA 1535	Pos[f]	Olajos and Cornish, 1976
	Escherichia coli Sd-B(TC)	Pos[f]	Nakajima *et al.*, 1974
Mammalian somatic cells in culture	Chinese hamster V79 lung cells	Pos[f]	Kuroki *et al.*, 1977
Chromosomal Mutations			
Mammalian cells in culture	Chinese hamster CHL cells	Pos[a]	Matsuoka *et al.*, 1979
Primary DNA Damage			
Yeast mitotic recombination and/or gene conversion	*Saccharomyces cerevisiae* D3	Pos[f]	Brusick and Mayer, 1973

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[a] No metabolic activation used.

[b] Strains tested with and without metabolic activation.

[c] Test material contained 87 ppm NDPA as a contaminant.

[d] Test for rII mutation

[e] Preliminary data - sample of test material with NDPA removed in the laboratory.

[f] *In vitro* chemical hydroxylation system used.

TABLE 9-3

Mutagenicity and Related Tests with Formulated Treflan (Part I) and Unspecified Forms of Trifluralin (Part II)

Part I: Treflan Formulations

Test Systems	Species/Strain	Result	References
Gene Mutations			
Bacterial	*Salmonella typhimurium* (8 strains)	Neg[a]	Anderson *et al*., 1972
Insect	*Drosophila melanogaster*	Neg[b,c]	Murnik, 1978
Chromosomal Mutations			
Insect	*Drosophila melanogaster*	Pos[b,c]	Murnik, 1978
Other studies	Exposed Humans	Pos[d]	Yoder *et al*., 1973
	Neurospora	Pos[c]	Griffiths, 1979
	Sordaria		Bond and McMillan, 1979
	Mouse bone marrow	Pos	Nehez *et al*., 1979

Part II: Unspecified Trifluralin

Gene Mutations			
Bacterial	*Salmonella typhimurium*[a]	Neg	Shirasu *et al*., 1976
	TA 1535	Neg	Shirasu *et al*. 1976
	TA 1536	Neg	Shirasu *et al*., 1976
	TA 1537	Neg	Shirasu *et al*., 1976
	TA 1538	Neg	Shirasu *et al*., 1976
	Escherichia coli[a]		
	B/r WP2 her$^+$	Neg	Shirasu *et al*., 1976
	WP2 her$^-$	Neg	Shirasu *et al*., 1976
Chromosomal Mutations			
Plants	*Haemanthus katheriniae*	[e]	Jackson and Stetler, 1973
	Tradescantia paludosa	[f]	Sawamura and Jackson, 1968
	Vicia faba	[f]	Sawamura and Jackson, 1968
	Triticum aestivum	Pos[g]	Bartels and Hilton, 1973
	Zea mays	Pos[g]	Bartels and Hilton, 1973
Salamanders	*Triturus helveticus*	[g]	Sentein, 1977
	Pleurodeles waltl	[g]	Sentein, 1977
Primary DNA Damage			
DNA Repair, Bacteria	*Bacillus subtilis* H17(Rec$^+$)/M45(Rec$^-$)	Neg[a]	Shirasu *et al*., 1976

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[a] Metabolic activation not used.
[b] Inconclusive results.
[c] Product sample used contained 177 ppm NDPA.
[d] Chromatid lesions in lymphocytes of workers exposed to herbicides.
[e] Decreased number of microtubules, accumulation of large vesicles at the cell plate region.
[f] Disruption of mitotic process temporarily impeded chromosome movement.
[g] Inhibition of mitosis as a consequence of spindle disruption.

Bacterial Tests. Simmon et al. (1977) tested 20 pesticides, including technical trifluralin (90%) containing 87 ppm NDPA as a contaminant in reversion-type mutagenic assays. Four Salmonella typhimurium strains and the WP-2 strain of E. coli were used, both with and without mammalian metabolic activation systems. Activation was obtained by using liver preparations from rats pretreated with polychlorinated biphenyl (PCB) Arochlor 1254. Trifluralin produced negative results in this study.

Simmon et al. (1977) also tested the same trifluralin sample for unscheduled DNA synthesis in human fibroblasts (WI-38 cells), mitotic recombination in the yeast Saccharomyces cerevisiae strain D3, and preferential toxicity in repair-deficient strains of E. coli and Bacillus subtilis, as compared to strains that could repair DNA damage. Each of these assays was performed both with and without mammalian metabolic activation over a wide range of trifluralin concentrations. Findings for trifluralin were negative in all assays. The experimental and data-reporting procedures used in this study appear to have been adequate.

Anderson et al. (1972) evaluated 109 herbicides, including both technical and formulated (44.5%) trifluralin. Specifically, the investigaters looked for induction of point mutations in a battery of standard bacterial and viral plate assays involving base air substitution and frameshift reversions as well as forward mutation. They compared their results to positive results with known mutagens specific for each of four assays. Single doses of all test chemicals, including formulated trifluralin (20 or 25 μg/plate), did not induce changes significantly different from the spontaneous rates of mutation in eight histidine-requiring mutants of Salmonella typhimurium, two rII mutants of bacteriophage T_4, or E. coli strain KB. This study did not conclusively demonstrate lack of mutagenicity because the investigators provided no exogenous metabolic activation to mimic possible conversion of the chemical to potentially active intermediates and they administered only one dose.

Shirasu et al. (1976) studied the mutagenicity of an unspecified form of trifluralin in four histidine-requiring strains of Salmonella typhimurium in a standard Ames assay as well as in differential toxicity assays with Bacillus subtilis strains H17 (Rec^+) and M45 (Rec^-) and in reversion assays with two strains of E. coli that require tryptophan. In each of these assays, the investigators treated bacterial cultures with a single saturated paper disc containing a 0.02 ml solution of a standard sample made up at a concentration of 1 mg trifluralin per milliliter of dimethylsulfoxide. Trifluralin showed negative results for mutagenicity in this study. However,the investigators did not provide mammalian metabolic activation, and they tested only one concentration. Thus, these results are inconclusive.

Insect Studies. Preliminary results from a study of both genetic and chromosomal effects in Drosophila melanogaster (Murnik, 1978 and I. Mauer, EPA, personal communication, 1978) showed "no evidence ... that trifluralin induces point mutations in *Drosophila*," but some of the results of these two separate studies are contradictory. In one portion of the first study, larvae were fed a diet containing 0.01% formulated trifluralin (44.5% AI) throughout their stages of development, and the number of sex-linked deaths was recorded in the F_2 (second) generation. In replicate tests, no significant differences were found in mortality rates between the treated (0.10% for the first test) and combined control (untreated spontaneous, 0.12%) groups. No positive controls were reported, and the formulated trifluralin tested contained approximately 177 ppm NDPA. In the second portion, adult males fed 0.02% of the formulated trifluralin for 2 days also incurred no increase in sex-linked deaths.

Although preliminary results from the two studies showed no evidence that trifluralin induces point mutations in *Drosophila*, Murnik (1978) reported an increase in chromosomal nondisjunction. Her first study involved the chronic feeding of 0.01% formulated trifluralin throughout the larval stages. Feeding at this stage resulted in a significant increase (0.12%) of XXY males compared to those of a control population (0.04%). However, feeding 0.02% formulated trifluralin to adult male *Drosophila* for 2 days resulted in no increase in nondisjunction. XXY nondisjunction was the only chromosomal aberration reported in this test. There were no increases over controls in chromosomal loss (XO progeny) or breakage. When the chromosomal portion of the first study was repeated, with technical trifluralin having no detectable NDPA, nondisjunction was not observed in test animals at a level significantly different from that of the control population. Thus, the cytogenetic results from these two studies are inconclusive as well as contradictory (Chaisson, 1978).

Studies with Fungi. Trifluralin has also been tested for nondisjunction in Neurospora crassa (Griffiths, 1979) as well as in Sordaria brevicollis (Bond and McMillan, 1979). Griffiths concluded that trifluralin produced aneuploidy in Neurospora. Nondose-related increases were found over a dosage range of 1-75 mg trifluralin/liter of culture. No information was given on the purity or source of the trifluralin nor of its ability to induce other genetic effects in Neurospora. The authors considered the results obtained in Sordaria to be inconclusive.

Rodent Tests. Male mice were injected with a trifluralin formulation containing 26% trifluralin (OLITREF®) in the following dosage regimen: A single dose of 200 mg/kg, two 100 mg/kg doses four 50 mg/kg doses, and a single 0.6 mg/kg dose. The fractional doses were administered at 3-day intervals (Nehez et al., 1979). Significant increases in chromosomal aberrations in the bone marrow were observed at all but the lowest dosage regimen. The LD_{50} of the trifluralin formulation was 600 mg/kg. The authors concluded that this preparation induced chromosomal aberrations in bone marrow at total doses of as little as one-third the LD_{50}.

Plant Studies. A number of plant studies have been conducted to determine whether trifluralin can disrupt the cellular spindle apparatus. The studies were not performed specifically to assess the issue of mutagenicity. In an in vitro and ultrastructural study of cellwall free endosperm cells of the African blood lily (Haemanthus katherinae), Jackson and Stetler (1973) reported that concentrations of trifluralin, ranging from 0.1-100.0 ppb, inhibited the rate at which cells progressed through all stages of mitosis from prophase to cell plate appearance. They observed these effects by time-lapse phase microscopy during a 2-hour period. Since 0.1 ppb had a near-maximum inhibitory effect, the data presented from all concentrations were pooled. Electron microscopic studies showed a decreased number of microtobules and an accumulation of large vesicles in the cell plate region.

The ultrastructural and mitotic index studies appear to have been conducted according to established protocols, but the bioassay used to assess these effects is not well documented. Furthermore, Jackson and Stetler did not establish a dose-response relationship, included no positive control in the study, and provided no information on the amount of NDPA that contaminated the study material. Nonetheless, this study does indicate that trifluralin interferes with the formation and function of plant cell microtubules, and the substance, may therefore, disrupt the mitotic spindle, thereby inducing numerical chromosomal aberrations.

Sawamura and Jackson (1968) treated staminal hair cells of the tetraploid Tradescantia paludosa and leaf cells of Vicia faba with 0.2 to 1.6 ppb of trifluralin. The degree of NDPA contamination for this material was unknown. At the highest dose (1.6 ppb), the authors reported the appearance of "dicentric bridges" in late stages of mitosis (anaphase and telophase) in both cell types, and cell elongation in staminal hair cells only. This study demonstrates that trifluralin can disrupt various stages of plant cell mitosis; however, the report is of limited value because the system tested is questionable, the data are not quantitative, and the study was not designed to assess mutagenicity.

Bartels and Hilton (1973) treated wheat (Triticum aestivum L., C.I. 5303) and corn (Zea mays L., Yellow Dent U.S. 13) with technical-grade trifluralin at 10^{-4}M. Cell division in the roots of the germinating seedlings appeared to be arrested at metaphase. The arrested cells showed no microtubule formation. Trifluralin did not inhibit microtubule assembly nor did it bind to the microtubule protein as does colchicine. The investigators concluded that trifluralin acts on the microtubular organizing centers, rather than on the microtubules *per se*.

Salamander Study. Sentein (1977) reported that trifluralin inhibited mitosis by interfering with the spindle apparatus in two urodele salamanders, *Triturus helveticus* and *Pleurodeles waltl*. Eggs of these species were incubated in various concentrations (1/8 through full saturation) of an unspecified form of trifluralin for 1 to 10 mitotic cycles prior to the beginning of cleavage, or at the 2-, 4-, 8-, and 16-blastomere stages. Cytologic observations were made during treatment and after various periods of incubation following transfer of the eggs to a trifluralin-free culture medium. At similar concentrations, the effects were more severe in

Pleurodeles than in Triturus eggs, but multinucleate blastomeres and disorganized mitotic figures occurred in both species. Sentein also reported disturbances in chromosomal condensation, especially at chromosomal sites associated with the mitotic spindle attachments, and gaps (discontinuities) at prophase. According to the author, the cytologic effects of trifluralin resembled those induced by classic antimitotic agents, but trifluralin was much less potent. The author concluded that these effects demonstrate that trifluralin interferes with the formation or function of cellular microtubular elements.

This study is difficult to interpret because of the lack of details pertaining to its protocol. For example, the source and composition of the trifluralin were not stated, nor were control data included (except that the solvent was reported to be polyethylene glycol). However, the study does confirm, in an animal test system, the potential antimitotic action of trifluralin previously found in plant cytologic studies.

The cytologic studies also support the genetic studies in Neurospora and Drosophila that indicate possible nondisjunctional activity of trifluralin (Mauer, 1978).

Human Survey. Yoder et al. (1973) observed chromosomal alterations in lymphocyte cultures prepared from samples obtained from people who apply pesticides. Blood was drawn once during the midwinter lull in spraying operations and again during the peak summer spraying period. Forty-two white male workers, who had from

1 to 25 years (mean exposure, 8.5 years) of prior occupational exposure to a variety of pesticides, were matched as closely as possible in age and physical characteristics to a control group of 16 businessmen, students, and teachers with no history of involvement with pesticides. The exposed group was divided into two subgroups. One consisted of 16 people who had been exposed to a variety of 17 insecticides; the other consisted of 26 employees of weed control agencies who had been exposed to 14 herbicides (most frequently to 2,4-D, amitrole, and atrazine, but also to formulated trifluralin).

The incidence of chromatid lesions per person in the worker groups increased significantly over that in the control group, but only in blood samples taken in the summer. Although Yoder *et al*. observed no heteroploidy (which may be indicative of nondisjunction) in any of the exposed or control cells, the authors did note a small number of chromatid exchange figures among the exposed groups. This study must be regarded as inconclusive in implicating trifluralin as a chromosome breaker, because it was only one of many pesticides used by these workers.

NDPA Mutagenicity Data

The principal contaminant of technical trifluralin preparations is NDPA, which is a demonstrated oncogen in rodents (Montesano and Bartsch, 1976). NDPA has been studied in *in vitro* mutagenicity assays with bacteria and yeasts, as well as in mammalian cell culture, coupled with appropriate mammalian metabolic activation systems (see Table 9-2). In bacterial assays, NDPA has caused reverse mutations by base-pair substitution at concentrations of up to 1.0 mmol, but only in the presence of complete liver enzyme preparations from rodents. When the cofactors for the microsomal mixed-function oxidase were omitted, the mutagenic effect was absent. Positive results for gene mutation as well as for chromosomal aberrations were also obtained in Chinese hamster lung cell cultures treated with 20 mmol NDPA and a rat liver enzyme preparation (Kuroki et al., 1977; Matsuoka et al., 1979).

Krueger (1973) also found direct evidence that NDPA alters genetic material in vivo. He reported the presence of alkylated guanine residues in DNA of rats given NDPA.

Trifluralin Derivatives

The mutagenic potential of degradation and/or metabolic products of trifluralin has also been assessed. Evidence indicates that trifluralin may degrade into a series of products, including substituted benzimidazoles in a mammalian-derived in vitro microsome system (Nelson et al., 1977). Such conversion has been reported to occur under ultraviolet photodecomposition conditions, especially in the vapor phase above treated soil, as well as in the soil. This finding is of concern because some benzimidazoles have been shown to be mutagenic (Seiler, 1972).

A report by Nelson et al. (1977) presents some interim results from bacterial mutagenicity assays performed with nine trifluralin metabolites, including some benzimidazoles. In these analysis, the investigators used plate incorporation at concentrations of up to 200 µg/plate for a standard battery of five test strains of S. typhimurium, both with and without metabolic activation. Summarizing the results, Nelson reported that he had found "no potent mutagens among these trifluralin derivatives tested thus far," as compared to the expected response of positive controls appropriate to each of the test strains.

Mutagenic Risk Assessment

Neither technical nor formulated trifluralin (containing NDPA at levels of up to 177 ppm) has shown any mutagenic activity in the studies cited here. The principal contaminant, NDPA at concentrations greater than 20 times those contained in current formulations of trifluralin (< 1.0 ppm), has induced mutations in various test systems (Mauer, 1978). NDPA is therefore considered to be a mutagen.

At least two situations of potential mutagenic risk exist: The direct effects on DNA and genes related to the NDPA contaminant, and the potential effects trifluralin induces on the spindle apparatus.

DNA and Gene Effects. When tests were performed with metabolic activation, technical trifluralin (containing approximately 87 ppm NDPA) did not produce significant gene mutations or primary DNA damage. Formulated trifluralin (as Treflan or unspecified) also produced negative results in some of the same tests; however, the results of these latter tests are inconclusive because they were performed without exogenous metabolic activation. Other preliminary studies indicate that Treflan, containing 177 ppm NDPA, as well as trifluralin with no detectable NDPA, produce negative results in the *Drosophila* sex-linked recessive lethal test. On the other hand, NDPA by itself has been shown to be mutagenic in several *in vitro* microbial test systems by causing base-pair substitution and primary DNA damage (Chaisson and Burkhalter, 1978). NDPA concentrations in the trifluralin preparations tested may have been too low to produce gene mutations or direct DNA interaction, especially in the presence of trifluralin (Chaisson and Burkhalter, 1978).

Trifluralin-NDPA mutagenicity data are not adequate to determine, much less to quantify, any risk for gene or DNA interactions posed by trifluralin. Any potential DNA and gene effects are associated with the NDPA contaminant of trifluralin formulations. To pose a potential, heritable genetic risk to humans, a chemical must be mutagenic and must be capable of reaching mammalian germ cells in a metabolically active form. There is no evidence showing whether mutagenically active forms of trifluralin or NDPA do or do not reach mammalian germinal tissue or whether these compounds are metabolized *in situ* to active forms if they do reach these tissues. The NDPA data in Table 9-2 indicate the need for metabolic activation of this compound before it can induce mutagenic responses in test organisms. Although NDPA has mutagenic activity in some *in vitro* test systems, including mammalian cells in culture, no *in vivo* tests have been performed.

Data on the structurally related aliphatic nitrosamines, dimethylnitrosamine (DMN), and diethylnitrosamine (DEN) can be used to bridge the information deficits concerning the *in vivo* mutagenic activity of NDPA. DMN and DEN are mutagenic in both the Ames *S. typhimurium* and *Drosophila* sex-linked lethal tests. Three mouse dominant lethal studies on these chemicals have also been performed. A single intraperitoneal dose of DEN (13.5 mg/kg body weight) did not significantly increase the number of mutations in the offspring of treated males (Propping *et al*., 1972). DMN also produced negative results when male mice were administered DEN at 8 or 9 mg/kg body weight by the same route (Epstein *et al*., 1972). DMN was reported to produce a weak dominant lethal effect in a second study with male mice (Propping *et al*., 1972). The second study has positive results with a DMN dosage lower than that yielding a negative response in the first study; however, the mouse strain and route of administration differed. Propping *et al*. used only a single treatment group. The lack of varying treatment levels precluded any within-experiment replication of the results or knowledge of dose-response relationships. The authors did state that the 4.4 mg/kg dose was the highest dosage of DMN compatible with survival.

Because of the great variation in the responses of animals in the dominant lethal test and the positive finding at a level of significance just meeting the accepted critical level, the study seems inconclusive. At face value, it suggests that DMN can reach the mammalian gonad. A negative interpretation, however, is consistent with the finding that neither DMN nor DEN stimulated unscheduled DNA synthesis in the mouse testis following intraperitoneal administration of the test compounds and tritiated thymidine (Gary Sega, personal communication, 1979). Also, DEN produced negative results in a specific locus test with mice (Russell, 1977). NDPA itself has not been tested for germinal or *in vivo* mammalian mutation. The available evidence does not support that Treflan-containing NDPA causes a significant risk to DNA and genes for the following reasons:

- NDPA appears to have point mutagenic activity in some *in vitro* systems, but information is lacking from *in vivo* tests. Some other short-chain alkylnitrosamines have been reported to produced positive results in the *Drosophila* sex-linked recessive lethal test.

- There is no direct evidence that NDPA does or does not reach the mammalian gonad in a genetically active form. As for other nitrosamines, it has been reported that neither DMN nor DEN stimulates unscheduled DNA synthesis in the mouse testis. Only one of three dominant lethal studies with these chemicals in mice suggests a positive effect, and that study reported a very weak positive finding with DMN. However, DEN produced negative results in a specific locus test with mice.

- Testing of trifluralin products containing 87 ppm NDPA has shown negative results for both mutagenic and DNA-damaging activity.

- A preliminary study with Treflan containing 177 ppm NDPA produced negative results in the _Drosophila_ sex-linked recessive lethal test.
- Exposure of humans to ND[illegible]hrough trifluralin use is expected to be very low.

At this time, it is not possible to quantify the mutagenic hazard that might be associated with the use of trifluralin contaminated with NDPA because information on the presence of the active compound in the mammalian gonad and the results of germinal testing are lacking. Occupational exposures to NDPA (5.05 μg/yr) and exposures of the general population through consumption of treated food (approximately 1.92×10^{-9} mg/kg body weight/day assuming the presence of a residue and a 5 ppm level of NDPA contamination in Treflan) are very low. Further, even plants and runoff water from fields treated with trifluralin (containing NDPA) did not contain any NDPA. The manufacturer has already lowered the contamination to 1 ppm or less. Thus, any risk is reduced further by a factor of approximately five.

Because risks of adverse effects are intimately related to exposure and because the expected exposure of humans to NDPA is low, it is also expected that any risk from point mutagenic effects would be mininal.

To obtain a better evaluation of point mutagenic risks, other tests should be conducted on NDPA, including studies assessing its ability to reach the mammalian gonad in a metabolically active form.

Spindle Effects. The limited studies that have been conducted appear to show that high concentrations of trifluralin (with or without stated levels of NDPA) can disrupt formation or function of the spindle apparatus in dividing cells, and thus have the potential to cause abnormal segregation of chromosomes (nondisjunction). Tests with formulated trifluralin (containing approximately 177 ppm NDPA) in *Drosophila* showed nondisjunction. Replication of these tests with technical trifluralin having no detectable NDPA produced negative results. However, positive results, showing effects on the spindle, were reported when formulated trifluralin (NDPA content unknown) was tested on *Neurospora*.

The positive chromosomal effects reported in plants and salamanders indicate that trifluralin (or trifluralin plus NDPA) may affect spindle fibers by interfering with microtubule formation or function. However no comparable studies in mammalian test systems, either *in vitro* or *in vivo*, have been reported. Because the mechanism of cell division does not differ significantly between plants and animals, similar spindle effects might be expected to occur in mammals exposed to trifluralin.

With this in mind, the committee surveyed mammalian and fish studies for evidence of mitotic disturbances, abnormalities in treated embryos, or any other chromosomal, spindle, or cellular effect of trifluralin on developmental processes. Overt manifestations of such effects include depressed cell formation and maturation, decreased viability of embryos, high resorption rates, or delayed tissue maturation (such as slow rates of ossification in neonates). Reports of vertebral hypertrophy in treated fish (Couch et al., 1978) and variations in skeletal development in mice (Beck, 1977) are not evidence of mitotic spindle effects and do not support the theory of a mutagenic effect of trifluralin in mammalian systems. Evaluation of hematologic values from chronic toxicity studies also did not elicit any such evidence (Mauer, 1978).

Thus, several lines of evidence from both the plant and animal kingdoms suggest that trifluralin products, containing known or unknown levels of NDPA, can interfere with the cell division spindle. Mammalian somatic or germinal cells have not been studied, but mammalian cells would probably respond similarly to cells of other organisms.

The existing data regarding effects on the cell division spindle at estimated trifluralin or NDPA exposure levels are inadequate for an assessment of risk. Furthermore, it is not clear whether trifluralin itself, one of its metabolites, or a contaminant is the active cause of the noted effects. Additional studies are needed to clarify these uncertainties.

CONCLUSIONS

Extensive mutagenicity testing has been performed with trifluralin, with positive results in some instances. These results are tainted because they were obtained using undefined trifluralin or trifluralin contaminated with the known mutagen-carcinogen NDPA at levels as high as 177 ppm. Therefore, it is quite possible that the mutagenicity (and carcinogenicity) attributed to trifluralin could be related to the contaminant. NDPA-free trifluralin should not be considered mutagenic (or carcinogenic) until a carcinogenesis bioassay on NDPA-free trifluralin is performed for comparison with the existing NCI study.

The majority of the mutagenicity studies with trifluralin produced negative results. Those that were positive for chromosomal damage and aneuploidy may be due to the presence of NDPA. Parallel studies with NDPA have not been reported. NDPA-free trifluralin and pure NDPA need to be tested, in tandem, for their abilities to induce chromosomal damage and aneuploidy.

Examination of the mutagenicity data on trifluralin preparations (including the formulated product) containing NDPA reveals an inadequate data base on which to evaluate potential hazards to DNA and spindles in laboratory animals or humans. NDPA may induce mutagenic effects, but the expected low exposures to this chemical suggest that the degree of hazard, even if NDPA are a germinal mutagen, are low.

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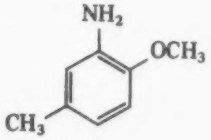
Chapter 10

p-CRESIDINE



p-Cresidine (2-methoxy-5-methylanaline) is a white crystalline solid that melts at 51°C. It is sparingly soluble in water, but volatilizes in the presence of steam. p-Cresidine is also known as 2-methoxy-5-methylbenzeneamine, 5-methyl-o-anisidine and, m-amino-p-cresol methyl ether.

p-Cresidine is obtained from the methylation and reduction by hydrogen of p-cresol, which is derived from the action of nitrous acid and excess nitric acid on p-toluidine.

PRODUCTION

Currently, the sole U.S. producer of p-cresidine is the Sherwin-Williams Company in St. Bernard, Ohio (Stanford Research Institute International, 1979). There is no record of plant capacity.

The production of p-cresidine was reported by the U.S. International Trade Commission in 1976-1977, implying that commercial production was greater than 2,300 kg/year (U.S. International Trade Commission, 1978). At least 450 kg/year was produced commercially in the United States in 1978-1979 (Stanford Research Institute International, 1979). Imports (in thousands of kilograms) through principal U.S. customs districts for 1976 to 1979 are as follows: 1976, 262.3; 1977, 40.2; and 1978, 125.7 (U.S. International Trade Commission, 1977, 1978, 1979).

USES

p-Cresidine appears to be used solely as a chemical intermediate in the production of dyes. The most commercially important of these dyes is the food, drug, and cosmetic dye (FD&C) Red No. 40, (of which 864 metric tons were produced in the United States during 1978 by the following companies: Buffalo Color Corp., Buffalo, N. Y.; Crompton & Knowles Corp., Gibraltar, Pa.; H. Kohnstamm & Co., Inc., Brooklyn, N. Y.; Hilton Davis Chemical Co., Division of Sterling Drug, Inc., Cincinnati, Ohio; and Warner-Jenkinson Co., St. Louis, Mo. (U.S. International Trade Commission, 1979).

p-Cresidine can also be used to manufacture six other dyes produced commercially in the United States, although no separate production figures were reported: C. I. Direct Blue 67, produced by Crompton & Knowles Corp., Fairlawn, N. J.; Direct Blue 126, produced by Harshaw Chemical Co., subsidiary of Gulf Oil Corp., Louisville Ky.; Direct Green 26, produced by Toms River Chemical Corp., Toms River, N. J.; Direct Orange 34, produced by Crompton & Knowles Corp., Fairlawn, N. J. and E. I. du Pont de Nemours & Co., Inc., Deepwater, N. J.; and Direct Red 79 and Direct Violet 9, produced by

Crompton & Knowles Corp, Fairlawn, N. J., and Toms River Chemical Corp., Toms River, N. J. (Colour Index, 1971; U.S. International Trade Commission, 1979).

EXPOSURE

There are few data to support an estimation of human exposure to p-cresidine. Small quantities of the chemical appear to be produced and imported and most exposures probably occur during the synthesis of dyes that use p-cresidine as an intermediate.

Some p-cresidine may appear as an impurity in FD&C Red No. 40. There are no Food and Drug Administration (FDA) regulations for p-cresidine; however, the FDA regulations for Red No. 40 allow for the presence of up to 1% each of sulfonated subsidiary dyes used in its production (21 CFR 74). The subsidiary dyes may result from the presence of impurities, such as p-cresidine, in the FD&C Red No. 40 intermediates, one of which is diazotized cresidine-p-sulfonic acid (Bell, 1976).

ANALYTIC METHODS

Although p-cresidine should respond to many of the procedures for primary aromatic amines, no information was available concerning analytic chemical methods for the compound. Sherwin Williams Company kindly provided the following information in the ensuing paragraphs (private communication, 1980).

Assays for Purity

Analysis is accomplished by using a gas chromatograph equipped with 1.5 m long column (0.32 cm inner diameter) packed with 20% SE-30 on Chromosorb W AW DMCS (80-100 mesh) and a flame ionization detector. The column oven is operated at 180°C and the injector and detector at 300°C; the nitrogen carrier flows at 20 ml/minute. Under these conditions, a methanol solution (0.4 µl) containing 400 µg of the compound is injected into the instrument for analysis; quantification is based on the area under the p-cresidine peak. Although the retention time (t_R) cannot be determined from the available information, it appears to be approximately 6 minutes or less. The peak is symmetrical. The sensitivity of the assay can easily be enhanced by a factor of approximately 1,000 by injecting a larger volume and using less attenuation; however, this technique offers no particular advantage to the purity analysis.

Analysis of Atmosphere in the Workroom.

Atmosphere in a Sherwin-Williams workroom is sampled by using an apparatus consisting of a midget bubbler charged with 10 ml of acetic anhydride followed by an activated carbon impinger and a membrane filter to protect the vacuum pump. Air samples are collected at a rate of approximately 1 l/minute for 1 to 2 hours. Any p-cresidine in the air reacts with the acetic anhydride to form the corresponding acetamide, which is analyzed by high-pressure liquid chromatography (HPLC).

The acetic anhydride from the bubbler is diluted to 25 ml with the same solvent, and 10 µl is injected into an HPLC equipped with a column of Partisil 1025 ODS (0.46 X 25 cm) and a UV absorption detector set at 280 nm. The mobile phase consists of 55% methanol-45% water containing 0.5% acetic acid; the flow rate is 1.0 ml/minute. Under these conditions the t_R of p-cresidine acetamide is approximately 7.0 minutes. Quantitation is accomplished by relating the areas under the peaks from samples of unknown p-cresidine content to those of standards of acetylamino-p-cresidine. The minimum detectable level of the compound in the diluted solution from the bubbler is 0.5 µg/ml.

HEALTH EFFECTS

Data on the toxicity of p-cresidine in animals and humans are virtually nonexistent. The National Institute for Occupational Safety and Health (1976) has reported an oral LD_{50} in rats of 1,450 mg/kg.

In dose range-finding studies conducted by the National Cancer Institute (NCI) Bioassay Program (National Cancer Institute, 1979) groups of five male and female F-344 rats and five male and female B6C3F1 mice were placed on diets containing 0, 1 and 3% p-cresidine for 8 weeks. Deaths (number unspecified) occurred in male and female mice and in female rats receiving the 3% diet. No deaths occurred in the male rats receiving 3% p-cresidine in any of the groups receiving the 1% diet or in the control group.

Chronic Toxicity

Carcinogenicity.

In the NCI Bioassay Program, p-cresidine was administered to male and female F-344 rats at concentrations of 0.5% and 1.0% in the diet for 104 weeks. The chemical was also administered for 104 weeks at time-weighted concentrations of 0.22% and 0.44% to female B6C3F1 mice for 104 weeks at a time-weighted concentration of 0.22% to male mice of the same strain and for 92 weeks at a time-weighted concentration of 0.46% to a separate group of male B6C3F1 mice.

Bladder carcinomas and olfactory neuroblastomas were observed in dosed rats of both sexes. A statistically significant incidence of neoplastic nodules of the liver, hepatocellular carcinomas, or mixed hepatocholangiocarcinomas also occurred in the low-dose male rats.

A statistically significant number of bladder carcinomas were also observed in both high- and low-dose male and female mice. In addition, both high- and low-dose female mice had a significant incidence rate of hepatocellular carcinoma.

Comparisons of p-cresidine to other single-ring aromatic amines and to 2-FAA (N-2-fluorenylacetamide) for potency in producing urinary bladder tumors in rats and mice are shown in Figures 10-1 and 10-2.

Figure 10-1 A comparison of the potency of single-ring aromatic amines and 2-FAA in producing bladder tumors in rats.

FIGURE 10-1

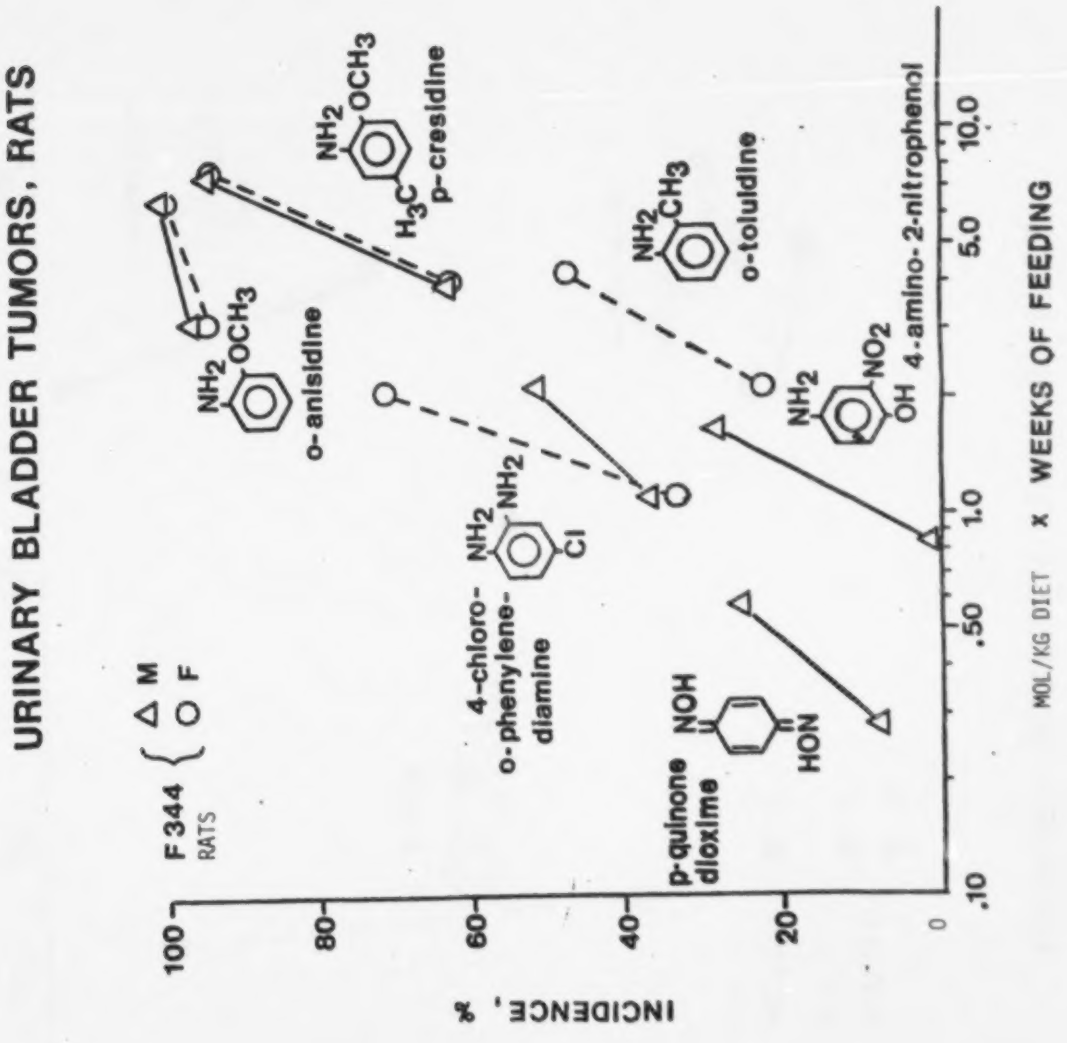


FIGURE 10-2

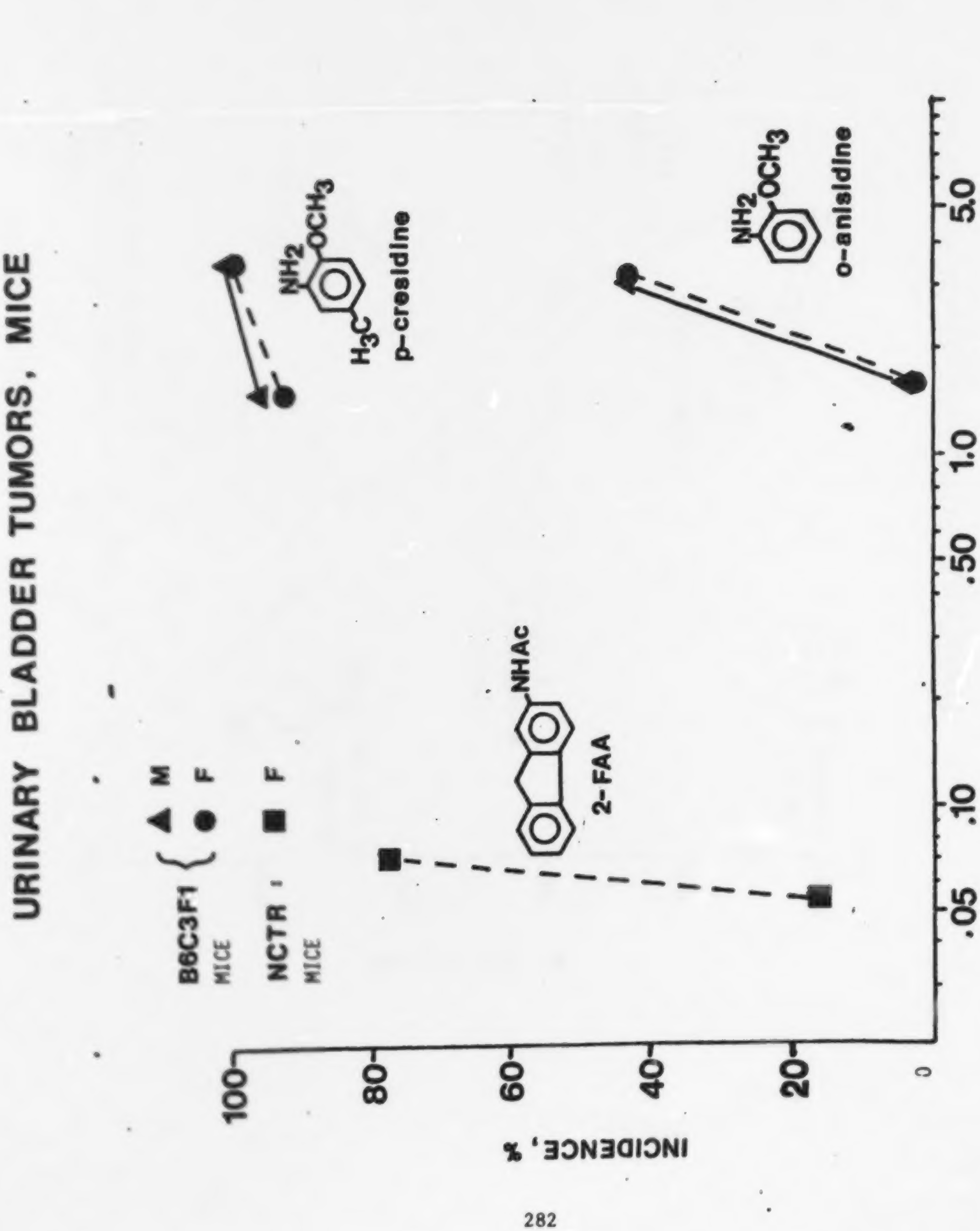


Figure 10-2 A comparison of the potency of single-ring aromatic amines and 2-FAA in producing bladder tumors in mice.

Mutagenicity

The only data on the mutagenicity of *p*-cresidine were obtained from the FDA Bureau of Foods. The results were from tests conducted by Stanford Research Institute International and Inveresk Research International (IRI) in the NCI collaborative study evaluating the reproducibility of results obtained in the *Salmonella* microsome and *Escherichia coli*, WP2 uvrA assays. The data from four *Salmonella* strains and one *E. coli* strain WP2 uvrA show that there are differences among the responses obtained by the two laboratories. For example, at IRI, *p*-cresidine induced mutations in TA 1537, TA 1538, TA 98, and TA 100 strains without metabolic activation. Similar differences for other chemicals were also obtained in this collaborative study. Overall, however, a positive response was reported by both laboratories for *p*-cresidine, with mutagenic dose-responses in TA 1538, TA 98, and TA 100.

Teratogenicity

No data were available to evaluate the teratogenicity or reproductive toxicity of *p*-cresidine.

CONCLUSIONS AND RECOMMENDATIONS

Other than the cancer bioassay conducted by NCI, there is virtually no other biologic data on *p*-cresidine. The lack of data makes it difficult to assess the potential health effects of this compound. Because of the positive carcinogenicity demonstrated in rats and mice, p-cresidine must also be considered potentially carcinogenic in humans. The preliminary mutagenicity data appear to show a positive response for p-cresidine, but confirmation is needed. Additional data are also needed on metabolism, metabolic activation, mutagenicity and genetic toxicity in both animal and human *in vitro* test systems.

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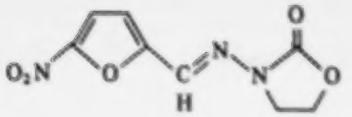
Chapter 11

FURAZOLIDONE



Furazolidone occurs as an odorless, yellow crystals that melt at 275°C. The crystals will darken under strong light and are decomposed by alkali. Furazolidone's solubility in water (pH 6) is approximately 40 mg/l. It is also known by the following synonyms and trade names: 3-[[(5-nitro-2-furanyl)methylene]amino]-2-oxazolidinone, 3-(5-nitrofurfurylideneamino)-2-oxazolidinone, N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone, NF 180, Furovag, Furoxane, Furoxone, Giarlam, Giardil, Medaron, Neftin, Nicolen, Nifulidone, Ortazol, Roptazol, Tikofuran, and Topazone. Furazolidone is produced synthetically from furfural, hydroxyethylhydrazine, and diethyl carbonate.

PRODUCTION

The sole U.S. producer of furazolidone is the Norwich Pharmaceutical Company in Norwich, N.Y. (Stanford Research Institute International 1979). The compound is prepared according to procedures described in U.S. patents 2,759,931 and 2,927,110 to Norwich Pharmaceutical Co. It was first produced in 1953 as a veterinary medicinal and feed additive, and in 1957 as a human systemic medicinal for U.S. and worldwide use (Federal Register, 1976a,b; Bryan, 1978).

The U.S. International Trade Commission (1976-1978) reports furazolidone data obtained on group of 20 pharmaceutical chemicals whose combined production totals approximately 3.2 million kg/year. The fact that production is reported to and by the commission indicates that annual production is 450 kg or more.

USES

Furazolidone is one of five 5-nitrofurans currently approved for use as systemic veterinary medicines in the United States (Federal Register, 1976a,b; Bryan, 1978). Its use was approved in 1953 to treat turkeys and chickens for fowl typhoid, paratyphoid, and pullorum; blackhead (histomoniasis); nonspecific enteritis (blue comb, mud fever), ulcerative enteritis (quail disease), and synovitis (arthritis due to filterable virus); and paracolon infection (*Paracolobactrum*). Furazolidone use is permitted in chickens for infectious hepatitis and coccidiosis, in turkeys for hexamitiasis, and in swine for bacterial enteritis (necrotic enteritis, black scours) or vibrionic (bloody) dysentery (Federal Register, 1976a).

Furazolidone is one of two 5-nitrofurans that have been approved in the United States as veterinary feed additives (Federal Register, 1976a,b), and it accounts for approximately 97% of the 5-nitrofurans administered to food-producing animals (Federal Register, 1976b). As a feed additive, the compound has been approved for use in chickens and turkeys to prevent fowl typhoid, paratyphoid, pullorum, air-sac infection and paracolon infection, and to enhance growth and feed efficiency. In swine, it is approved for the prevention of bacterial enteritis and vibrionic dysentery, and for growth promotion and enhanced feed efficiency (Federal Register, 1976a). In 1976, the Food and Drug Administration (FDA) published proposals to withdraw approval of furazolidone for the veterinary purposes for which it is now used (Federal Register, 1976a,b). To date, no final action on these proposals has been taken.

Furazolidone has been used in humans to treat bacillary dysentery, typhoid and paratyphoid fevers, giardiasis, brucellosis, and intestinal infections of undetermined etiology (Bryan, 1978; Miura and Reckendorf, 1967; Paul and Paul, 1964, 1966).

EXPOSURE

Furazolidone has been the subject of controversy in recent years. The chemical has been determined to cause cancer when ingested by rats and mice, although the producer has challenged this finding. In 1976, the FDA proposed to withdraw approval for use of the drug in food-producing animals (Federal Register, 1976a). At that time, the agency ruled that data were not adequate to determine the total drug-related residues that can occur, that the analytic techniques for measuring the drug were not reliable for the lower concentrations found in food, that the drug was present in edible tissue following medication when no withdrawal period was observed, and that a reliable withdrawal period could not be established from the information available.

The results of testing for furazolidone in controlled experiments are shown in Table 11-1. As indicated, each study concluded that residues were undetectable (usually with a detection limit of 1 to 5 mg/g) after a 2- to 19-day withdrawal period.

The 1976 proposal to ban the use of furazolidone in food-producing animals was withdrawn because of problems in obtaining supportive data, but another proposal is being issued in 1980 (Moy, Food Animal Additive Branch, FDA, personal communication, 1980). Although FDA requires that food products contain no residue, compliance is handled by the U.S. Department of Agriculture. Because analytic techniques for sampling at low concentrations are unreliable, the existing regulations have not been enforced.

It is not possible to estimate the degree to which humans are exposed to this drug. The largest potential for exposure occurs from its presence in food. However, the residue disappears or, at the very least, becomes undetectable, within 20 days after the withdrawal of medication. Thus, the concentrations expected in furazolidone-treated food products cannot be estimated. Moreover, there is no information concerning the percentage of the total poultry and swine treated with the drug. Minute quantities may be released to the air, water, and solid waste in the vicinity of the plant.

Table 11-1

Results of Testing for Furazolidone in Food

Food Type	Dose	Residue	Reference
Chicken	"Excessive"	None after 2 days	Krieg and Loeliger, 1973
Eggs	100 mg/100 ml water for 3 days, and 5 mg/100 mg for 2 days	8 mg/g on 3rd day; none after 10 days	Krieg, 1972
Eggs	40 mg/100 g water for 15 days	23 micrograms/g on 6th day; none after 19th day	Krieg, 1972
Veal	Unknown	None found	Nouws, 1973

ANALYTIC METHODS

Most of the analytic methods for furazolidone are based on thin-layer chromatography (TLC), high-pressure liquid chromatography (HPLC), UV absorption spectrophotometry, or combinations of these techniques.

A general procedure using TLC to identify 18 drugs, including furazolidone, in animal feed was reported by Williams (1978). The sample was extracted with acetonitrile-chloroform (4:1), and an aliquot of the extract was cleaned on a column of aluminum oxide. The concentrated eluate was then subjected to TLC on silica gel G by using chloroform-methanol (9:1) to develop the plate. Spots were made visible on the plates by spraying with 1,2-diaminoethane or with Dragendorff's reagent.

Cieri (1978) also reported a method for determining furazolidone in animal feed. The sample was extracted with acetone and cleaned on TLC plates of silica gel H by using chloroform-methanol (9:1) to develop the plates. The appropriate zone was scraped from the plate, eluted with ethyl alcohol, and quantitated at its absorption maximum near 360 nm. The method was reported not to be applicable at levels below 0.005%.

Moretain et al. (1979) described a procedure for determining furazolidone and furaltadone in admixture at the levels expected in animal feed (i.e., 50 to 200 ppm). The sample was extracted with dimethylformamide (DMF) and subjected to TLC on silica gel plates developed with chloroform-acetone (7:3). The appropriate zones were then scraped from the plates, extracted with DMF, and determined spectrophotometrically at 370 nm.

Methods reported recently have all been based on HPLC. Lefebvre (1979) demonstrated that furazolidone could be assayed by HPLC, using a glassy carbon or carbon paste electrode, coupled with a voltametric-amperometric detector. Detection limits were reported to be in the low nanogram range; however, no actual residue assays were performed. Using HPLC Jones et al. (1978) determined furazolidone levels in swine and poultry feed at levels as low as 5ppm, with a silica column and a mobile phase of water-saturated dichloromethone. The UV-visible detector was set at 360 nm. The sample preparation consisted only of extracting the feed with methanol and 2 N hydrochloric acid (1:1), partitioning the residue into dichloromethane, and concentrating the solvent for a 20 µl injection into the HPLC system. Again using HPLC, Cieri (1979) determined residues of furazolidone and nitrofurazone in feeds at levels as low as 0.5 ppm. The sample was extracted with DMF-acetone (1:1), cleaned on a column of silica gel, and analyzed on a reverse-phase column with 30% acetonitrile as the mobile phase. The detector was set at 365 nm.

Hoener et al. (1979) recently reported an HPLC procedure for determining residues of furazolidone in turkey tissue at levels as low as 2 ppb. The tissue was ground with methanol and centrifuged.

The extract then was either injected directly or concentrated before being injected into the HPLC system. A Bondapak C_{18} column was used with a mobile phase consisting of methanol and 0.01 mol sodium acetate (1:4). The UV absorption detector was set at 365 nm. Recoveries from muscle and liver spiked at the 2 ppb level were 103 ± 19% and 112 ± 12%, respectively.

Bagon (1979) used a Spherisorb S5-ODS column for HPLC separations of several antibiotics and nitrofurans in pharmaceutical preparations. Furazolidone was separated from nitrofurazone by using a mobile phase of water-methanol (11:9) with the absorption detector set at 375 nm. Although no samples other than pharmaceutical preparations were analyzed, the procedure is said to be generally capable of distinguishing the parent compounds from decomposition products and likely congeners.

Problems associated with the analysis of furazolidone in edible tissues of at levels of 0.5 to 4.0 ppb with methods available prior to 1976 are well documented (Federal Register, 1976). Various spectrophotometric and TLC procedures failed to yield satisfactory and reproducible recoveries at these levels.

HEALTH EFFECTS

Metabolism

Furazolidone is biotransformed *in vivo* in mammals to a variable but major extent (Swaminathan and Lower, 1978). Two to five metabolites have been suggested from *in vitro* or *in vivo* analyses (Federal Register, 1976a; Tatsumi *et al*., 1978). One biotransformation product, 3-(4-cyano-2-oxobutylideneamino)-2-oxazolidone, was characterized by mass-, ultraviolet-, and nuclear-magnetic-resonance-spectroscopic methods following furazolidone incubation *in vitro* with milk xanthine oxidase or administration *in vivo* to rabbits, when it was identified in urine. In contrast to furazolidone, this metabolite was not an active mutagen in *Salmonella typhimurium* TA 100 (Tatsumi *et al*., 1978).

Acute Toxicity

Humans. Reported symptons of acute toxicity of furazolidone in humans include nausea, emesis, occasional diarrhea, abdominal pain, and bleeding (Cohen, 1978). Infrequently, there have been reports of an Antabuse-like reaction to alcohol (Cohen, 1978) or, rarely, idiosyncratic or hypersensitivity reactions such as pneumonitis (Cohen, 1978; Collins and Thomas, 1973; Cortez and Pankey, 1972; Jirasek and Kalensky, 1975). Hemolytic anemia due to glucose-6-phosphate- dehydrogenase deficiency has been reported with furazolidone (Cohen, 1978). Furazolidone demonstrated monoamine-oxidase inhibition (Pettinger and Oates, 1968) and required precautions when coadministered with other monoamine-oxidase inhibitors, sympathomimetic amines, or tyramine-containing foods. No data have been reported concerning chronic toxic effects of furazolidone in humans.

Animals. Furazolidone induced emesis and neurologic changes in dogs (Miura and Rekkendorf, 1967; Rogers _et_ _al_., 1956). No published oral LD_{50} data exist for mammals. The drug also induced cardiomyopathy in turkeys (Czarnecki _et_ _al_., 1978; Staley _et_ _al_., 1978).

Chronic Toxicity

Carcinogenicity

Humans. No furazolidone-associated carcinogenicity in humans has been reported.

Animals. Furazolidone was evaluated for carcinogenic activity in six studies in rats and one study in mice (Federal Register, 1976a,b; Cohen, 1978), but no review of these studies has yet appeared. However, the FDA has summarized the statistically significant effects resulting from its analyses of the data submitted (Federal Register, 1976a,b) and has used these evaluations as the basis for proposed regulatory action (Federal Register, 1976a,b). Statistical comparisons of significant effects in rats and mice of both sexes exposed to varying dose levels of furazolidone administered orally for 18 or more months are presented in Tables 11-2 through 11-5.

TABLE 11-2

Statistical Comparison of Significant Effects Produced in Groups of 50 Male and Female Sprague-Dawley Rats Fed Furazolidone[a]

	Dose of Furazolidone, ppm[b]							
	Males				Females			
Effect	0	250	500	1,000	0	250	500	1,000
Malignant mammary tumors								<0.05[c]
Benign mammary tumors						<0.2	<0.01	
Mammary tumors						<0.05	<0.05	<0.05
Multiple mammary tumors						<0.05	<0.05	
Nonmammary tumors[d]				<0.05				
Mortality, 18 months			<0.05	<0.05				<0.05
Mortality, 26 months				<0.05			<0.01	<0.02

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[a] Abstracted from Federal Register, 1976a.

[b] Fed diet for approximately 18 months (553 days); surviving rats maintained on furazolidone-free diet until mortality in each group reached 90%, at which time the remaining 10% were killed approximately 13 months after furazolidone was withdrawn from the diet.

[c] Value of P compared to rats fed furazolidone-free diet.

[d] Included squamous cell carcinoma, dermal fibroma, pituitary neoplasms, leukemia, and lymphosarcoma.

TABLE 11-3

Statistical Comparison of Significant Effects Produced in Groups of 50
Male and Female Fischer 344 Rats Fed Furazolidone[a]

	Dose of Furazolidone, ppm[b]							
	Males				Females			
Effect	0	250	500	1,000	0	250	500	1,000
Malignant mammary tumors								<0.05[c]
Mammary tumors						<0.01	<0.01	<0.01
Multiple mammary tumors						<0.01	<0.01	<0.01
Thyroid adenomas			<0.05	<0.05			<0.05	<0.05
Sebaceous adenomas			<0.05	<0.05				<0.05
Testicular mesotheliomas				<0.05				
Basal cell epitheliomas				<0.05				
Mortality			<0.05	<0.05				

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[a] Abstracted from Federal Register, 1976a.

[b] Fed diet for 20 months; surviving rats maintained on furazolidione-free diet until mortality in each group reached 90%, at which time the remaining 10% were killed approximately 11 months after furazolidone was withdrawn from the diet.

[c] Value of P compared to rats fed furazolidone-free diet.

TABLE 11-4

Statistical Comparison of Significant Effects Produced in Groups of 40 Male and Female Sprague-Dowley Rats Fed Low-Levels of Furazolidone[a]

	mg/kg/day x 265 days[b] (ppm beyond 266 days)							
	Males				Females			
	0	1	15	15	0	1	5	15
Effect	(0)	(25)	(125)	(375)	(0)	(25)	(125)	(375)
No. of rats with tumors								<0.05[c,d]
Multiple mammary tumors							<0.05	<0.05
Onset of palpable mammary tumors, 16 months								<0.05
Mortality, 20 months								<0.05
Mortality, 24 months							<0.05	<0.05

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[a] Abstracted from Federal Register, 1976a.

[b] Fed diet for 265 days on basis of mg/kg of body weight/day; thereafter until termination of study at 2 years (731 days) on basis of ppm in diet.

[c] Value of P compared to rats fed furazolidone-free diet.

[d] Majority of tumors were in mammary gland; in addition there were pituitary adenomas and lymphoreticular neoplasms, including leukemia, lymphosarcoma, and reticulum cell sarcoma.

TABLE 11-5

Statistical Comparison of Significant Effects Produced in Groups of 50
Male and Female Swiss MBR/ICR Mice Fed Furazolidone[a]

	Dose of Furazolidone, ppm							
	Males				Females			
Effect	0	75	150	300	0	75	150	300
Bronchial adenocarcinoma			<0.05	<0.05				<0.05
Malignant tumors			<0.05	<0.05				<0.05
Benign plus malignant tumors			<0.05	<0.05				<0.05
Multiple tissue tumors				<0.05				
Mortality				<0.05			<0.05	<0.05

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[a] Abstracted from Federal Register, 1976a.

[b] Fed diet for 18 months; surviving mice maintained on furazolidone-free diet for an additional 10 months at which time study was terminated.

Furazolidone was fed to 35 female Sprague-Dawley (Holtzman strain) rats for 45 weeks followed by a drug-free diet for 8 additional weeks before the rats were sacrificed. A control group of 35 rats was fed a drug-free diet for 53 weeks. Rats fed furazolidone had a significantly higher incidence of mammary tumors than did control rats (Federal Register, 1976a).

In second study, 20 male and 20 female rats (CFE strain) were fed furazolidone for 45 weeks. The animals then received a drug-free diet for 7 more weeks. Control groups of both sexes were fed a drug-free diet for the 52 weeks. The female rats fed furazolidone had a higher incidence of mammary tumors than did the female controls. No significant effects on tumor development were noted in male rats (Federal Register, 1976a)

A third 2-year evaluation of furazolidone toxicity was also reported (Federal Register, 1976a) for 60 rats divided into three groups, each consisting of 10 males and 10 females. One group of each sex was fed a furazolidone-free control diet. The diets for the other groups contained two levels of furazolidone. There were three times as many tumors in rats fed furazolidone at a level of 0.01% in the diet as compared to the number of tumors in the control group (Federal Register, 1976a).

A fourth study involved 2-month-old male and female Sprague-Dawley (Charles River strain) rats fed furazolidone (Table 11-2). The number of female rats with malignant mammary tumors increased as the dosage level increased, and the dose-response relationship was reported as linear and significant ($\underline{P} < 0.01$). At the highest dose level (1,000 ppm), the proportion of female rats with malignant tumors was significantly higher ($\underline{P} < 0.05$) than that of the control rats fed the furazolidone-free diet. Furazolidone also concurrently induced benign mammary tumors in female rats. Furazolidone did not induce mammary tumors in male rats; however, it did randomly induce tumors in other body tissues. The proportion of male rats that developed tumors of other tissues at the 1,000 ppm dose level was significantly higher ($\underline{P} < 0.05$) than that of controls. Finally, furazolidone-treated male and female rats exhibited a drug-related early mortality (Federal Register, 1976a).

A fifth study in which furazolidone was fed to 2-month-old male and female Fischer 344 rats is summarized in Table 11-3. The proportion of female rats with malignant mammary tumors at the 1,000-ppm level was significantly higher ($\underline{P} < 0.05$) than that of control rats. Malignant mammary tumors were diagnosed only in female rats fed the highest dosage of furazolidone. At all dosage levels, the proportion of female rats with mammary tumors was significantly higher ($\underline{P} < 0.01$) than that of control rats. A significant relationship between the dosage of furazolidone and the proportion of female rats with mammary tumors was reported (Federal Register, 1976a). The proportion of female rats with multiple mammary tumors was significantly higher ($\underline{P} < 0.01$) for all treatment groups than in controls. Other tumors that occurred more significantly ($\underline{P} < 0.05$) in male and female rats fed furazolidone include thyroid and sebaceous adenomas (both sexes), testicular masotheliomas, and basal cell epitheliomas in male rats (Table 11-3). A dose-response (early mortality) relationship was significant for both male and female rats (Federal Register, 1976a).

A sixth study, in which furazolidone was fed to 2-month-old male and female Sprague-Dawley (Charles River strain) rats, is summarized in Table 11-4. There was a significant ($\underline{P}$ <0.05) linear dose-response relationship for the number of female rats with tumors, and a significant increase ($\underline{P}$ <0.05) in the proportion of female rats at the 375-ppm dose level with tumors, as compared to females fed a furazolidone-free diet. The types of tumors observed are shown in Table 11-4. A significant increase ($\underline{P}$ <0.05) of the incidence of multiple mammary tumors for both the 125- and 375-ppm dose groups (as compared to controls) was noted, with a significant ($\underline{P}$ <0.05) linear dose-response relationship. Furazolidone resulted in significant and early onset and development of mammary tumors in female rats at the 375-ppm dose level as compared with that of controls. There was a significant linear dose response for mammary tumor development in female rats during the first 16 months of the study. Early significant mortality ($\underline{P}$ <0.05), which was dose related, was reported for female rats. No significant tumor or mortality rate differences were found for male rats (Federal Register, 1976a).

A study of furazolidone fed to 2-month-old male and female Swiss MBR/ICR mice is summarized in Table 11-5. Bronchial adenocarcinomas were significantly higher ($\underline{P}$ <0.05) in both male and female mice than in controls fed a furazolidone-free diet. Incidence of this growth in both sexes showed a significant (_P_ <0.01) and linear dose response (Federal Register, 1976a). The dose-response relationships for malignancies of all tissue types as well as for the incidence of all tumors were significant (_P_ <0.05) and linear for both sexes. There was a significant increase (_P_ <0.05) both in the development of malignancies and in the development of benign and malignant tumors combined in male mice at the 150 and 300 ppm levels, and in female mice at the 300 ppm level, as compared with those of control mice. Male mice that received the 300 ppm dose had significantly more (_P_ <0.05) multiple tumors than did control mice. There was also a significant linear dose-response relationship for tumor multiplicities for female mice fed furazolidone. Male and female mice fed furazolidone both exhibited significant (_P_ <0.05) early mortality which was linear in dose response (Federal Register, 1976a).

Mutagenicity

The data from mutagenicity tests of furazolidone are summarized in Table 11-6. Furazolidone was found to be mutagenic in _Escherichia coli_ (Lu _et al_., 1979; McCalla and Voutsinos, 1974) and _Drosophila melanogaster_ (Blijleveh _et al_., 1977) caused chromosomal damage in human lymphocytes (Cohen and Sagi, 1979), and cross-linking of DNA in _Vibrio cholerae_ (Chatterjee _et al_., 1977).

TABLE 11-6

Mutagenicity Tests of Furazolidone

Test System	Species-Strain	Results	Reference
Gene Mutation			
Bacterial	*E. coli* - WP2 uvr A⁻	pos[a]	McCalla and Voutsinos, 1974
	E. coli - WP2,WP2 uvr A, CM561 (lex A), CM 571 (rec A), CM 611 (uvr A, rec A)	pos[a]	Lu *et al.*, 1979
Insect	*D. melanogaster* (Berlin-K, Oregon-K)	pos	Blijleven *et al.*, 1977
Chromosomal Damage			
Mamalian cells in culture	Human lymphocytes	neg	Tonomura and Sasaki 1973
	Human lymphocytes	pos	Cohen and Sagi, 1979
Bacterial	*Vibrio cholerae*	pos	Chatterjee *et al.*, 1977

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[a] Tester strains without metabolic activation.

Bacterial Tests. Furazolidone was among 22 nitrofurans that McCalla and Voutsinos (1974) found to be mutagenic in *E. coli* WP2 and its uvrA-derivative by reversion from trp^- to trp^+. ExrA-or recA tester strains were not induced to mutate by the nitrofurans, indicating that mutants arise when the lesions induced by these compounds are repaired by the "error-prone" repair system, components of which are coded for by *rec* and *exr* genes. Results obtained with the nfr-strains, which lack nitrofuran reductase I, suggest that the nitro group is a key structural component, and that only when the nitro group is reduced and the drug converted into a more reactive compound are mutants induced. In this respect, mutagenesis of nitrofurans is similar to the formation of alkali-labile DNA lesions by these compounds (McCalla *et al*., 1971).

The mutagenic activity of the nitrofurans so far tested covers a wide range (Lu *et al*., 1979, McCann *et al*., 1975). Lu *et al*. (1979) reported an approximately 10,000-fold range in mutagenicity of eight nitrofurans, including furazolidone. The most active compound was 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, (AF-2) which was approximately 6 times as mutagenic as furazolidone. Lu *et al*. (1979) ranked these compounds from most to least toxic to *E. coli* as follows: AF-2 > *N*-[(5-nitro-2-furyl)thiazolyl]formamide (FANFT) > 2-amino-4-(5-nitro-2-furyl)thiazole (ANFT) > furazolidone 1-[[3-(5-nitro-2-furanyl)-2-propenylidine]amino]-2,4-imidazolidinedione (furagin) > nitrofurazone > methylnitrofuroate > nitrofuroate. In general, mutagenic activity was found to parallel toxicity.

Insect Tests. The genetic effects of furazolidone were determined in *D. melanogaster* by the induction of sex-linked recessive lethals, which is the most sensitive mutation test in this organism (Blijleveh *et al*. 1977). Furazolidone was fed to adult males, which were then mated with fresh virgins for three consecutive periods of 3, 2, and 2 days. A consistent increase in the frequency of sex-linked recessive lethals was observed for furazolidone under these conditions, indicating that the spermatids are sensitive to this compound, as they were to AF-2 (Blijleven *et al*., 1977). Although the mutation frequency for furazolidone is low in this system (as in AF-2), and a demonstration of dose-response effect is lacking, the present data indicate that furazolidone and AF-2 are mutagenic in *Drosophila*.

Chromosomal Damage

Mammalian Cells in Culture. The capacity of furazolidone to induce chromosomal damage (chromosomal breaks, sister-chromatid exchange, DNA repair synthesis, and inhibition of mitosis) in cultured human peripheral lymphocytes was examined by Tonomura and Sasaki (1973) and Cohen and Sagi (1979). Tomomura and Sasaki (1973) did not report either a significant amount of chromosomal abberation or unscheduled DNA synthesis for furazolidone over an 0.5 to 100 x 10^{-6} M dose range. In contrast, Cohen and Sagi (1979) found that furazolidone produced dose-dependent mitotic suppression, chromosomal breakage, and sister-chromatid exchanges (SCE's). The different results are surprising and difficult to explain, in that the experimental design of the two studies (e.g., range of drug concentration used, exposure times, solvents, methods of cytologic scoring) were similar. However, the fact that furazolidone induced dose-dependent SCE in the Cohen and Sagi study (1979) and that this type of chromosomal damage was not reported by Tomomura and Sasaki (1973) suggests that this compound is capable of inducing chromosomal damage in human cells. Further studies are needed to clarify this point.

Bacterial DNA. Furazolidone inhibits DNA synthesis in *V. cholerae* cells while stimulating RNA and protein synthesis and causing filamentation of these cells (Chatterjee and Maiti, 1973). Chatterjee *et al*. (1977) reported that interstrand cross-linking in DNA takes place within the furazolidone-treated *V. cholerae* cells, which therefore might explain the actual mechanism of inhibition of DNA biosynthesis by this drug. The *in vivo* action of furazolidone has considerable similarity to that of mitomycin C (Iyer and Szybalski, 1964). Both agents induce interstrand cross-linking in DNA, inhibit DNA synthesis, and cause filamentation of the cells at the appropriate dose level by inhibiting cell division.

Teratogenicity

No data were available to evaluate the teratogenicity or embryotoxicity of furazolidone.

CONCLUSIONS

Furazolidone has exhibited carcinogenic effects in male and female rats and mice in a variety of tissues. Susceptible tissues are different for the two species. Furazolidone, in common with most 5-nitrofurans studied, is a significant carcinogen in rodents (Cohen, 1978).

Furazolidone is highly mutagenic in both microbial (*E. coli*) and insect (*D. melanogaster*) test systems, produces chromosomal damage (breakage, SCE, mitotic suppression) in human lymphocytes, and forms interstrand cross-linking in bacterial (*V. cholere*) DNA.

It is for the above reasons that the use of furazolidone is now being reviewed by the FDA. Resolution of this matter awaits the development of a sufficiently sensitive and reliable analytic method. If risk to human health does exist, it most certainly would be associated with the use of furazolidone for veterinary purposes. A "solution" may involve the substitution of an efficacious product that could be demonstrated not to have the mutagenic and carcinogenic potential of furazolidone.

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